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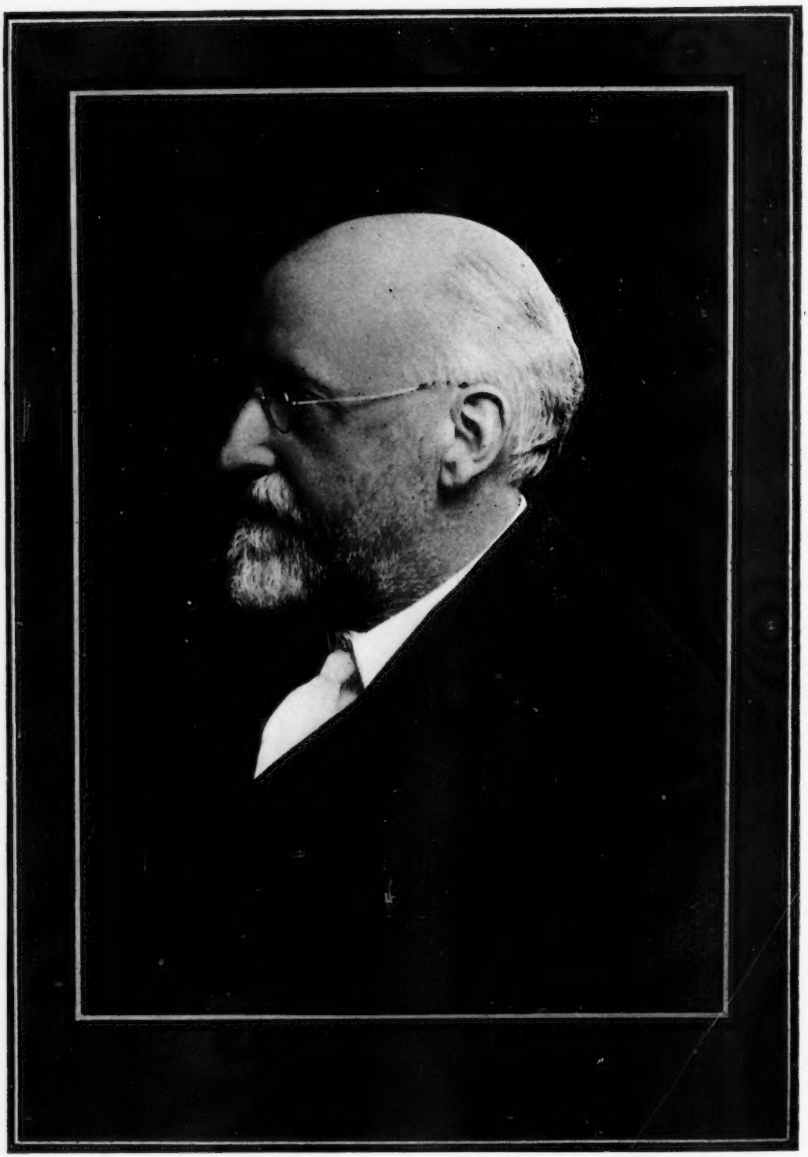


Photo.] THE LATE *[Elliott & Fry.*

SIR WILLIAM MADDOCK BAYLISS, F.R.S.,

Professor of General Physiology in University College, London.

THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE LATE

SIR WILLIAM MADDOCK BAYLISS, F.R.S.

As has been announced in the Press, Sir William Bayliss died on August 27th after a long illness.

The published work of Sir William Bayliss is so well known and admired that we feel it to be superfluous to attempt an account of it here. We wish, however, to acknowledge the debt which this Journal owes to him.

When we had under consideration the project of founding this Journal, Sir William Bayliss was one of the first to whom we applied for advice, and it was largely his approval which encouraged us to proceed.

Sir William Bayliss did not confine himself to moral support. He was active in providing us with papers by his friends and pupils, and he himself contributed the first article we published. Further, his interest was not only manifested in this respect, but in the generosity with which he assumed with us the initial financial risks involved.

Thus as Editors of this Journal we have special cause to associate ourselves with the expressions of deep regret his death has occasioned.

THE EFFECT OF RADIATION ON THE PRODUCTION OF SPECIFIC ANTIBODIES.

PERCIVAL HARTLEY.

From the National Institute for Medical Research, London.

Received for publication September 19th, 1924.

In a recent communication, Colebrook, Eidinow and Hill (1924) have shown that exposure of rabbits, piglings and man to various types of radiation increases the bactericidal power of the blood against staphylococci and a streptococcus of the *fæcalis* type. This increased bactericidal power is essentially transient in nature; the maximum is reached in one to two hours and then declines rapidly to the original value. Further analysis of the phenomenon showed that both serum and leucocytes contributed to the effect. Exposure of the skin to radiation produces first an erythema and, subsequently, desquamation and pigmentation. The authors suggest that the absorption into the blood of the products of tissue damage, or the enhanced absorption of normal tissue products produced by such damage, and the consequent erythema and œdema, appears to be a natural excitant of immunizing mechanisms.

In view of these results it was of interest to see what the effect of such non-specific stimuli as exposure to the mercury vapour lamp or the carbon arc lamp would be on certain specific immunizing mechanisms. Guinea-pigs immunized against diphtheria toxin and rabbits immunized against *B. typhosus* have been used in the experiments described in this paper; the effect of exposure to radiation on the immune body content of the serum has been investigated. The inquiry was carried out at the suggestion of Dr. Leonard Hill. The attempt has been made to subject the experimental animals to the same conditions, as regards exposure to radiation, as those which were adopted by Colebrook, Eidinow and Hill. The exposures to the various types of radiation were carried out for me by Dr. Hill and his colleagues, and I desire to express my thanks to them for their help, and for the endeavour they have made to ensure that the experimental conditions were the same in these experiments as in those already reported by them.

A. THE EFFECT OF RADIATION ON THE DIPHTHERIA ANTITOXIN CONTENT OF THE SERUM OF IMMUNIZED GUINEA-PIGS.

Colebrook, Eidinow and Hill found that the increase in hæmo-bactericidal power reached a maximum very rapidly after irradiation. This result is of interest when considered in relation to the work of Walbum (1921) and Madsen (1923), who found that a single intravenous injection of manganous chloride was followed by a striking rise in antitoxin content, the maximum being reached in one hour and then falling slowly. Further, Salomonsen and Madsen (1898) found that injections of pilocarpine into immunized horses was

followed by an increase in antitoxin, which reached a maximum in about half-an-hour and then declined. McIntosh and Kingsbury (1924), however, were unable to confirm Walbum's results.

White animals, or animals with white flanks, were used in all radiation experiments. The flanks were shaved on the day previous to irradiation. As

TABLE 1.

Guinea pig.	22.1.24 Antitoxin units per c.c. before irradiation.	23.1.24 Irradiation.	23.1.24 Antitoxin units per c.c. 1 hour after irradiation	23.1.24 Antitoxin units per c.c. 3 hours after irradiation
5 A	0·02	Merc. Vap. Lamp for 30 min. at 9 inches.	0·02	0·02
5 B.	0·02	Merc. Vap. Lamp for 30 min. at 9 inches.	0·02	0·02
6A	0·04	Carb. Arc Lamp for 30 min. at 18 inches.	0·04	0·04
6B	0·002	Carb. Arc Lamp for 30 min. at 18 inches.	0·002	0·002

TABLE 2.

Guinea pig.	7.11.23 Antitoxin units per c.c. before irradiation.	8 & 9.11.23 Irradiation.	10.11.23 Antitoxin units per c.c. after irradiation.
3 B	0·2	Merc. Vap. Lamp for 20 min. at 18 inches on 8th.	0·2
8 A	0·2	Merc. Vap. Lamp for 30 min. at 18 inches on 9th.	0·2
3 A	0·8	Nil.	0·8
8 B.	0·6	Nil.	0·6

TABLE 3.

Guinea pig	5.12.23 Antitoxin units per c.c. 20 days after primary stimulus	6 & 7.12.23 Irradiation or other treatment	13.12.23 Antitoxin units per c.c. 6 days after irradiation.	13 & 14.12.23 Irradiation	19.12.23 Antitoxin units per c.c. 5 days after irradiation.
2A	<0·001	Nil.	<0·001	Nil.	0·001
2B.	<0·001	Nil.	0·001	Nil.	0·002
3A	<0·001	0·02 M.L.D. dip. toxin on 7th.	>0·01	Nil.	>1
3B.	<0·001	0·02 M.L.D. dip. toxin on 7th.	>0·01	Nil.	1
1A	<0·001	30 min M.V. Lamp on both days.	0·001	30 min M.V. Lamp on both days.	0·002
1B.	<0·001	30 min M.V. Lamp on both days.	0·001	30 min M.V. Lamp on both days.	0·001
4A	<0·001	30 min Carbon Arc on both dys.	<0·001	30 min Carbon Arc on both days.	<0·001
4B.	<0·001	30 min Carbon Arc on both days.	<0·001	30 min Carbon Arc on both days.	<0·001

in the experiments of Colebrook, Eidinow and Hill, exposure to radiation produced erythema and, subsequently, desquamation and pigmentation. Œdema, which was most pronounced on the day following irradiation, was an almost constant finding in animals exposed to the mercury vapour lamp.

Experiment 1.—The following experiment was carried out to see whether exposure to radiation was accompanied by an *immediate* increase in antitoxin. The results are given in Table I. The antitoxin content of the serum

remained unaltered one and three hours after exposure to the mercury vapour lamp and the carbon arc lamp.

Experiment 2.—The effect of two exposures to the mercury vapour lamp on successive days was investigated. Antitoxin determinations were made twenty-four hours before the first exposure and twenty-four hours after the second exposure. The results are shown in Table II. No change in antitoxin occurred either in the experimental or control animals.

Experiment 3.—Eight normal guinea-pigs were injected subcutaneously with 1 c.c. of toxin-antitoxin mixture. Nineteen days afterwards all animals were shaved, and on the following day they were bled and antitoxin determinations carried out. The amount in all cases was less than one-thousandth of a unit, and further attempts to estimate the actual quantity present were not made. Two animals (2A and 2B) received no further treatment beyond being bled at the same time as the remaining six; they served to study the effect of a single "primary stimulus" (Glenny and Sudmersen, 1921). Two other animals (3A and 3B) received subcutaneously, twenty days after the primary stimulus, one-fiftieth of a minimum lethal dose of diphtheria toxin as a "secondary stimulus." Beyond bleeding at the same time as the other animals of the series these received no further treatment; they served to study the effect of a specific secondary stimulus. Two other animals (1A and 1B) were exposed to the mercury vapour lamp on four occasions, and the remaining two animals (4A and 4B) were exposed to the carbon arc lamp; in these four animals the effect of non-specific stimuli—radiation—was investigated. The results of this experiment are collected in Table III.

The control guinea-pigs showed, as the result of the primary stimulus, a very small production of antitoxin at the end of thirty-four days. The two guinea-pigs which received the same primary stimulus, followed twenty days afterwards by a single small injection of toxin as a secondary (and specific) stimulus, responded typically by the production of relatively large quantities of antitoxin. Guinea-pigs receiving the same primary stimulus and—in place of toxin—four exposures to radiation, showed at the end of thirty-four days amounts of antitoxin which, in the case of the two animals exposed to the mercury vapour lamp, were identical with the control animals, while the two animals exposed to the carbon arc lamp showed no increase whatever in antitoxin during the experiment.

B. THE EFFECT OF RADIATION ON THE AGGLUTININ CONTENT OF THE SERUM OF RABBITS IMMUNIZED AGAINST *B. TYPHOSUS*.

The effect of exposure to the carbon arc lamp on the typhoid agglutinins has been investigated by Hansen (1922). His experiments were carried out on twenty-three patients suffering from lupus or surgical tuberculosis (mild cases). These were given 1 c.c. of typhoid vaccine, exposed to the action of the carbon arc lamp at different and for varying times after inoculation, and their agglutinin curves plotted. In nine cases the agglutinin curve was unaffected. In fourteen cases Hansen considers that exposure to light influenced the amount of agglutinins in the blood, since the curve in these cases showed either (1) a plateau in place of the usual peak, (2) a delayed fall in the curve, or (3) an actual rise in the curve after the fall had begun. Control experiments

on the agglutinin curve of non-irradiated lupus and surgical tuberculosis patients, similarly vaccinated against *B. typhosus*, are not recorded, and in the absence of such data it cannot be admitted that such variations in the agglutinin content of the serum as Hansen describes are of significance, or are to be ascribed to exposure to the carbon arc lamp. The American Trench Fever Commission (1918) examined a large number of *B. typhosus*-inoculated subjects (T.A.B. vaccine), and found that even in normal individuals the range of fluctuations in agglutinin titre may be large or small, or the titre may remain practically constant over a period of two months. The fluctuations recorded in normal individuals by the Commission were larger than those recorded by Hansen.

The effect of exposure to the carbon arc lamp of rabbits immunized against *B. typhosus* was also investigated by Hansen. He found that the agglutinin curves for both control and irradiated animals exhibited fluctuations, and he ascribes this as being due, in part at least, to repeated small venesections. He found, however, that whereas all six non-irradiated rabbits died from six to twenty-six days after inoculation, of the irradiated animals one died on the third day, one on the twenty-seventh day, and four survived for over two months. From his experiments on rabbits Hansen concludes that exposure to the carbon arc lamp enabled the animals to withstand the toxic effects of the killed typhoid culture.

In the experiments described below the effect was investigated of exposure to radiation, under conditions similar to those adopted by Colebrook, Eidinow and Hill, on the agglutinin content of the serum of rabbits immunized against *B. typhosus*.

Fourteen rabbits were injected intravenously with 1 c.c. of *B. typhosus* vaccine, the material used for this purpose being the standard agglutinable culture supplied by Prof. Dreyer, of the University of Oxford. There were no deaths; the animals showed no ill-effects from the inoculation and all increased in weight. The complete agglutinin curve of twelve of these animals was determined; in the remaining two cases the rising and early falling portions of the curve were not determined. Seven rabbits were used for investigating the effect of radiation on agglutinin content, and seven were used as controls to study the effect of repeated small bleedings on the agglutinin curve.

The animals were exposed to radiation at different stages of immunization, viz. (1) when the agglutinin curve was at, or near, its maximum; (2) when the curve was falling rapidly; (3) when the curve had declined to its lowest level, and was parallel, or nearly so, to the base-line.

The individual experiments were carried out in the following way: A sample bleeding was taken about 10 a.m. and an hour afterwards the animal was irradiated. A second bleeding was taken one hour, a third bleeding three hours, and a fourth bleeding twenty-four hours after irradiation. Other sample bleedings were taken several days later as shown on the charts. Thus, the immediate and late effects, if any, of irradiation could be determined. At the same time as the irradiation experiments were carried out one or more control animals, immunized but not irradiated and at the same stage of immunization, were bled at the same time, and, as far as one could judge, to the same extent. Thus, the effect of bleeding *alone* could be determined.

In considering this group of experiments it is convenient to review the results obtained with the control animals first. It is well known that hæmorrhage may result in an increase in antibodies in the serum of immunized animals (see O'Brien, 1913, who reviews the literature). Schroeder (1909) showed that a single bleeding of 25 c.c. in a rabbit was followed by a rise in agglutinin, and Reymann (1917) found in two rabbits immunized against *B. coli* that repeated small bleedings—3 c.c. daily, or in some cases every two days—was accompanied by a large increase in agglutinins, and this increase was maintained for sixty to seventy days.

The effect of repeated small bleedings at short intervals on the antibody content of the serum is shown on the accompanying charts (control animals: rabbits E, J, K, L, N, O and X). At each bleeding from 2·5 to 3 c.c. of blood were taken, so that in twenty-four hours the total amount of blood withdrawn was 10 to 12 c.c. The effect on the agglutinin content of the serum may be summarized as follows:

(1) In no case was an immediate increase in agglutinins observed.

(2) In four cases there was a slight but detectable fall in agglutinins after three small bleedings had been taken at short intervals on the same day. This fall was observed when the fourth small bleeding was taken on the following day.

(3) In two cases an increase in agglutinins was observed. This became manifest five days after the four repeated small bleedings. The actual increase in agglutinin titre was from $\frac{1}{1600}$ to $\frac{1}{2500}$ (rabbit E) and from $\frac{1}{500}$ to $\frac{1}{700}$ (rabbit J).

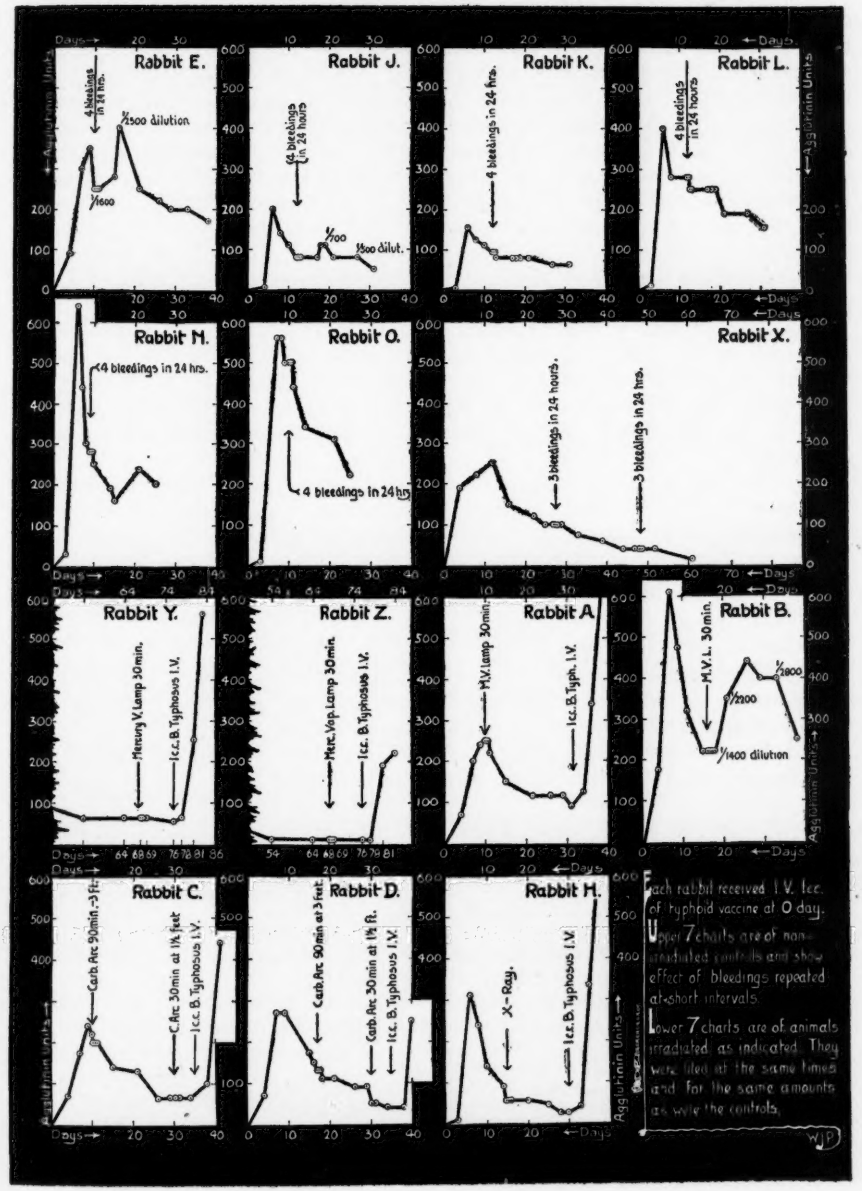
(4) In one case (rabbit N) the repeated bleedings had no effect for five days, but later the titre rose from $\frac{1}{1000}$ (fifth day) to $\frac{1}{1500}$ (eleventh day).

Thus, repeated small bleedings at short intervals—a procedure frequently necessary in immunological investigations—may produce an effect on antibody titre, although these effects may not be so marked as those which follow larger bleedings.

The possibility that the variations in agglutinin titre observed might be due to errors in technique was not overlooked. Dreyer's (1920) method was used, the same emulsion (standard agglutinable culture) being employed for any particular animal. Particular care was given to the preparation of the serum dilutions. It was expected that any change in agglutinin titre resulting from irradiation would not be large, and might, indeed, be missed if the ordinary method of doubling dilutions was used. The particularly important parts of the agglutinin curves are those showing the titre before and after irradiation, and these points were determined in duplicate—first, by the ordinary method of doubling dilutions to ascertain the approximate titre, and second, by making new dilutions by the drop method to cover the previously ascertained range. Small differences in the titre could thus be detected.

The results of exposure to radiation on the typhoid agglutinins of immunized rabbits may be summarized as follows:

(1) Four rabbits were exposed to the action of the mercury vapour lamp. One exposure only was given—for thirty minutes, distance 9 in. A good reaction followed exposure in all cases. In three cases—rabbits A, Y and Z—exposure produced no effect, either immediate or late, on the agglutinin


Rabbit E.
4 bleedings in 24 hrs.
1/1500 dilution
1/1600
Rabbit J.
4 bleedings in 24 hours
1/100
1/500 dilut.
Rabbit K.
4 bleedings in 24 hrs.
Rabbit L.
4 bleedings in 24 hours
Rabbit M.
4 bleedings in 24 hrs.
Rabbit O.
4 bleedings in 24 hrs
Rabbit X.
3 bleedings in 24 hours.
3 bleedings in 24 hrs.
Rabbit Y.
Mercury V. Lamp 30 min.
1 c.c. B. Typhosus I.V.
Rabbit Z.
Merc. Vap. Lamp 30 min.
1 c.c. B. Typhosus I.V.
Rabbit A.
M.V. Lamp 30 min.
1 c.c. B. Typh. I.V.
Rabbit B.
M.V.L. 30 min.
1/100
1/800
1/1400 dilution
Rabbit C.
Carb. Arc 90 min. 3 ft.
C. Arc 30 min at 1½ feet
1 c.c. B. Typhosus I.V.
Rabbit D.
Carb. Arc 90 min at 3 feet
Carb. Arc 30 min at 1½ ft.
1 c.c. B. Typhosus I.V.
Rabbit H.
X-Ray.
1 c.c. B. Typhosus I.V.
Agglutinin Units
Days
Each rabbit received 1 V. 1 c.c. of typhoid vaccine at 0 day.
Upper 7 charts are of non-irradiated controls and show effect of bleedings repeated at short intervals.
Lower 7 charts are of animals irradiated as indicated. They were bled at the same times and for the same amounts as were the controls.
W.P.

content of the serum. In each case a subsequent injection of *B. typhosus* emulsion (standard agglutinable culture) was followed by a typical response to the specific stimulus. In one case (rabbit B) no immediate increase to agglutinins followed exposure to radiation. On the fifth day after irradiation the titre had increased (from $\frac{1}{1400}$) to $\frac{1}{2200}$, and five days later it had increased still further to $\frac{1}{2800}$: the titre subsequently declined. It has been shown above that bleeding alone may be accompanied by an increase in agglutinins, and although the increase in the case of rabbit B is somewhat larger than the increases observed in the normal animals, the conclusion that the agglutinins have increased as a result of exposure to the mercury vapour lamp does not appear to be warranted. Further, in the case of rabbit B an extra small sample bleeding was taken—by inadvertence—forty-eight hours after exposure, and this may have contributed to the slightly greater effect observed.

(2) Two rabbits (C and D) were exposed to the carbon arc lamp for the time and at the distances shown on the charts. Exposure was followed by typical reactions after each exposure and the body temperature was increased by 2° to 3° F. The effect on the agglutinin content of the serum was *nil;* each animal responded typically to the specific stimulus—1 c.c. *B. typhosus* killed culture—subsequently.

(3) One animal (rabbit H) was exposed to the action of X-rays (Coolidge tube, spark-gap 8 in., focal distance 8 in., current 4–5 milliampères). There was no effect on the agglutinins. The result is in agreement with the work of Hektoen (1918), who investigated the action of X-rays on the production of lysins and precipitins in rats, rabbits and dogs.

CONCLUSIONS.

(1) Exposure to radiation, under the conditions outlined in the foregoing experiments, is without effect on the content of diphtheria antitoxin and typhoid agglutinin of the serum of immunized animals.

(2) Repeated small bleedings at short intervals may be followed by an increase in typhoid agglutinins in immunized rabbits.

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ŒDEMA IN SOUTH AFRICAN CATTLE.

ARTHUR DIGHTON STAMMERS.

From the Department of Physiology, University of the Witwatersrand, Johannesburg.

Forwarded for publication September 10th, 1924.

THREE cases of œdema have recently occurred among animals slaughtered at the Johannesburg Municipal Abattoir. The condition is not prevalent at this time of the year (July), but its incidence is generally increased during the later months, after the prolonged period of drought which usually ensues during June, July, August and September in the higher altitudes of South Africa. This fact indicates that the disease is nutritional in origin. It has been suggested that, in humans, the so-called "war œdema" was due to deficiency in vitamins, and that the particular factor concerned is vitamin A (McCarrison, 1919–20), although Madsen (1918) states that it may be a form of beri-beri.

On the other hand, Kohman (1920–21) considers that vitamin-deficiency plays little part in the production of œdema, and that a combination of low calories, low protein and excessive fluid intake is sufficient to produce the condition.

Friedberger and Frohner (1910) state that "chronic hydræmia of cattle is frequently observed in abattoirs as an œdema of cellular tissue, and is caused by long-continued feeding with food which contains too much water." In such cases, post-mortem examination reveals that "the entire muscular system is pale and flaccid. The subcutaneous and intermuscular connective tissue is infiltrated with serous fluid. The flesh is consequently watery, the cavities of the body are more or less filled with transudate, the viscera are shrivelled up, pale and devoid of fat and the brain is œdematous. We almost always observe signs of chronic intestinal catarrh."

Apart from these remarks, there appears to be little dealing with this subject in the literature.

The cases which have come before my notice appear to present certain significant features which are somewhat at variance with those detailed above. For example, the muscular system appeared normal in colour and condition. Infiltration was marked, but the viscera themselves were normal to naked-eye examination. The adipose tissue, however, was almost devoid of fat, which was replaced by a serous fluid. The bone-marrow was probably most abnormal of all the tissues, and presented the typical appearance of "gelatinous marrow," which is stated to be due to hunger and certain pathological conditions (Bohm and Davidoff, 1916; Piersol, 1920).

Upon inserting a knife into the medullary cavity of a long bone, this gelatinous marrow could be separated and removed, and, although it retained its shape, was most strikingly œdematous in appearance. This applies to the first case investigated; the others were not so abnormal, and in these the marrow had the usual set, yellow appearance, although there was evidence of a certain amount of transudation in the abdominal cavity and the adipose tissue was slightly œdematous.

The cattle having been condemned on account of their poor condition, it was decided to make an investigation into the chemical changes, if any, involved, and for this purpose portions of the marrow and adipose tissue were selected as being the most obviously affected. Naked-eye examination, as already mentioned, revealed little that was abnormal about the muscles or the bones, but portions of these were also removed for examination.

Analyses were conducted with the following results. Analyses of normal tissues are appended for purposes of comparison.

	Case 1. Œdema marked.	*Case* 2. Œdema slight.	*Case* 3. Œdema slight.	Normal cattle.
	Per cent.	Per cent.	Per cent.	
BONE.				
Moisture	31·32	17·22	15·62	9·00
Protein	14·69	16·35	17·38	23·30
Fat	0·47	0·86	1·10	2·00
Ash	52·79	65·20	65·30	66·00
Phosphoric oxide*	15·60	32·71	31·18	38·50
ADIPOSE TISSUE.				
Moisture	95·00	60·88		9·97
Fat	3·10	37·01	Not	88·37
Membrane	1·13	1·03	examined	1·66
Ash	0·61	0·59		—
BONE-MARROW.				
Moisture	85·28	20·16	20·61	—
Fat (ether ext.)	1·03	75·87	76·60	—
Organic residue	13·32	3·11	3·62	—
Ash	0·03	0·04	0·05	—

* Percentage of total ash.

Muscle.	*Case* 1. Œdema marked. Per cent.	*Case* 2. Œdema slight. Per cent.	*Case* 3. Œdema slight. Per cent.	Normal cattle.
Moisture	78·12	74·19		75·76
Protein	17·22	19·38	Not	20·12
Ether ext.	1·32	5·21	examined	3·00
Ash	0·89	0·68		1·12

On perusal these figures reveal striking discrepancies between the affected cattle and the normal. Dealing with bone first, in all three cases there is a very definite increase in moisture and a deficiency in protein and in fat. The increase in moisture in Case 1 (the most severe) is very marked.

In the case of the total ash there is a considerable diminution in Case 1, and there is also a still more marked and disproportionate decrease in the phosphorus content. This diminution also applies to Cases 2 and 3, but is not so noticeable in these.

As regards adipose tissue, Case 1 again yields the most abnormal figures, replacement of fat by moisture here being very noticeable. Case 2 is also noteworthy, but the differences in this are less marked.

In the case of bone-marrow, the diminution of fat in Case 1 is also very well seen, and there is a distinct decrease in the protein content of the muscle in this case.

The author has recently endeavoured to produce a condition resembling that seen in the cattle in rats by feeding on a diet deficient in phosphates, but adequate otherwise. The rats were fed upon a ration consisting of—

Purified caseinogen	22·10 per cent.
Starch	45·90 „
Cane-sugar	12·75 „
Refined cod-liver oil	15·00 „
Salt mixture	4·25 „

The salt mixture was prepared according to the following formula. The figures indicate parts in 500:

Sodium chloride	47·25
Magnesium sulphate	71·68
Calcium lactate	337·00
Ferric citrate	31·52
Sodium fluoride	0·55
Manganese sulphate	2·00
Potassium iodide	10·00

In practice the basal diet was made up with caseinogen, starch, sugar and salt mixture, and the day's food prepared according to requirements by incorporating the appropriate amount of fat and making up the whole to a pasty consistency with water. In addition, a small quantity of dried yeast was added to the food each day, so that the diet was adequate as regards its vitamin content (rats do not require vitamin C).

Two male animals were used, the technique otherwise being as described by the author (1921).

The experiment started on July 1st, 1924, the weights of the animals being then 212 and 199 gm. From that time the weights fell steadily. The experiment terminated on August 9th, when both died, the weights immediately prior to death being 124 and 110 gm. respectively. Post-mortem examination revealed that, in each case, the bone-marrow of the femur had become gelatinous, but otherwise no evidence of œdema could be seen. Both animals were markedly emaciated, and there was no observable subcutaneous or other fatty tissue.

In the case of one of the animals, a smear of the gelatinous marrow showed the presence of micrococci, which may have originated post-mortem, but otherwise nothing abnormal except the expected diminished numbers of fat-globules, which appeared somewhat œdematous, could be seen.

The amount of food eaten was carefully determined and averaged 8·25 gm. per animal per day, with but little variation from that figure.

DISCUSSION.

It will probably be agreed that the condition in the cattle examined resulted from malnutrition of one kind or another. During the periods of drought this is a common occurrence, and far more animals are affected than ever reach the abattoirs. The actual significance of the term " malnutrition " is a very wide one, and of the factors which may combine to produce the condition, the most important may be summarized as follows:

1. Actual deficiency in total amount of food ingested.
2. Deficiency in calorie value of food.
3. Improper balance of proximate principles.
4. Deficiency in one or more vitamins.
5. Deficiency in one or more proximate principles.

So far as our present knowledge takes us, malnutrition, so-called, must be due to the effect of the above factors, either singly or in combination.

It would be desirable to inquire as to what foods are available to cattle during times of drought, but this is somewhat beyond the scope of the present paper, although it is hoped, in a future investigation, that some information as to this may become available.

It has been demonstrated that most South African soils are deficient in phosphorus. Theiler, Green and Du Toit (1924) have emphasized the importance of this element in the live-stock industry, and any deficiency may be made up by feeding bone-meal. It is probable that, when the importance of this is fully recognized, a marked step forward in the elimination of nutritional diseases will have been accomplished.

So far as the experiment on rats described above applies, items 3 and 4 of the contributing factors previously mentioned may be dismissed, since the diet used conformed to requirements in this direction. Referring to items 1 and 2, the amount of food eaten was somewhat subnormal, but not excessively so, and it would seem that some other factor must be looked for. This may perhaps be found under item 5. It appears that there is some evidence that a diet satisfactory in other factors, but deficient in phosphorus,

tends to bring about the condition seen in the cattle referred to. It is true that, so far, the only comparable condition observed in the rats was the gelatinous bone-marrow and the marked emaciation. However, this, although by no means conclusively due to the lack of phosphorus, certainly points in that direction, and it remains for future experiments, which it is hoped may be carried out on larger animals, to decide the point.

I wish to express my indebtedness to Mr. A. C. Kirkpatrick, Assistant Director of the Johannesburg Municipal Abattoir, for kindly affording me facilities for obtaining specimens from affected cattle.

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THE EFFECTS OF LIGHT UPON LEUCOCYTES AND BLOOD-VESSELS IN THE MESENTERY OF THE LIVING ANIMAL.

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Evidence from recent researches supports the theory that the therapeutic action of light depends upon changes in the epidermis, and blood circulating in the skin, exposed to radiation. Hess and Gutman (1921) have demonstrated that the inorganic phosphorus of the serum, which is lowered in rickety children, returns to normal under the influence of sunlight treatment. Rollier (1921) states that in the treatment of surgical tuberculosis by heliotherapy good pigmentation of the skin and a good prognosis are accompanied by a lymphocytosis. In this connection Murphy and Sturm (1919) have found that mice with a high lymphocyte count are resistant to the inoculation of tubercle bacilli. Janet Clarke (1922) recently reviewed the literature regarding the physiological action of light and gives many interesting details. She states that the effect of sunlight on the erythrocyte count is not very marked, whereas the white blood-cells, especially the lymphocytes, respond to

short exposures of any radiation (sunlight, ultra-violet light, X-ray, heat). All published results agree that ultra-violet light stimulates a lymphocytosis in men and animals. The production of lymphocytosis as well as the formation of pigment may well be an important factor in heliotherapy. A lymphocytosis might result from an increased rate of production or from a decreased rate of destruction, probably the former. Janet Clarke states that presumably the lymphocyte-forming organs are stimulated to greater activity by some photochemical change produced by ultra-violet light. Whatever the change may be it probably takes place primarily in the epithelial cells which absorb the ultra-violet rays, and secondarily in the walls of the capillaries as the result of irritation of the epithelial cells.

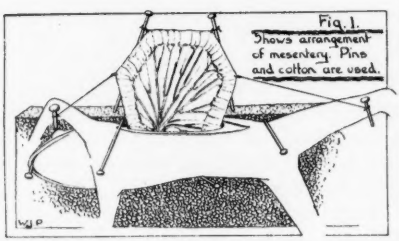
Colebrook, Eidinow and Leonard Hill (1924) showed that when the shaved skin of a rabbit is exposed to sunlight, ultra-violet light, radiant heat and certain other mildly irritant influences, the animal's blood quickly acquires an enhanced power of killing the ordinary pyogenic cocci—*e. g.* staphylococci and streptococci. Colebrook (1924) found that these changes in bactericidal activity in whole blood produced under the influence of sunlight are referable to changes in the functioning of the leucocytes. Hausmann (1910–11) sensitized mice to visible rays by injection of hæmatoporphyrin, and obtained definite leucocytosis in the skin, particularly near the surface muscle. He concluded that injury to the peripheral vessels in the skin is the primary trouble in the sensitization of mice by hæmatoporphyrin to visible rays. In our experiments recorded below we have obtained further evidence that light has an important action, direct or indirect, upon leucocytes of the blood. Before dealing with these results we describe the method and some experiments which led up to them.

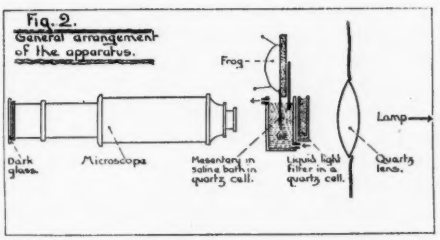
METHOD.

Finsen (1893) studied the effects of light upon the blood-vessels of a living animal by means of the microscope. He found that radiations from the sun produced dilatation of capillaries, emigration of leucocytes and stasis in the tadpole's tail. Dreyer and Jansen (1905) employed the same tissue and also the frog's web, tongue and mesentery to study capillary stasis resulting from radiation with ultra-violet light. They found the frog's tongue to be most convenient; it required less manipulation and less handling than the mesentery. We employed the mesentery (frog and mouse), since it was suited for our purpose and the necessary operation did not affect our conclusions. For anæsthesia, in the case of the frog, the cerebrum was crushed; when a mouse was used urethane was injected subcutaneously. The animal was fixed to a cork frog-board by means of ligatures and pins. Its body lay close to one edge of the board. A portion of the small intestine about 1 cm. in length was stretched gently by means of ligatures between two pins jutting out from the edge of the cork board (see Fig. 1). The ligatures were tied round a small area of the outer coats of the outer border of the gut—we found this more efficient than piercing the gut. The gut was in no way obstructed or twisted, so that the circulation in the portion of mesentery under investigation was quite free. The opening made in the side-wall of the abdomen was large enough to prevent compression of the exposed structures. By means of

ligatures suitably placed the portion of mesentery between the two pins was prevented from folding and other organs were similarly prevented from covering it.

The piece of gut with its mesentery was then dipped into a small saline bath (see Fig. 2), which was placed below the body of the animal so that the edge of the cork board rested on the sides of the bath. The bath was kept at a constant temperature by a steady stream of saline entering below from a reservoir and flowing away at the top of the bath. The wall of the saline bath exposed to the light was of quartz (3 mm. thick), and thus permeable to ultra-violet rays as well as to other rays. The mesentery was placed about 1 cm. behind this wall—that is, in the middle of the bath. When required the rays from the source of light were focussed on the mesentery by means of a suitable lens; also, when necessary, a fluid filter was employed to exclude one or other set of rays (see Fig. 2). As a source of rays we used two lamps, a mercury vapour lamp (Kelvin, Bottomley and Baird's 2·5 amp.) and





a carbon arc lamp (25 amp., 85–100 volts). The circulation in the mesentery was under constant observation by means of a microscope, the eye-glass of which was covered with coloured glass to protect the eye of the observer.

EXPERIMENTS WITH THE MERCURY VAPOUR LAMP.

Influence of temperature.—Leonard Hill and Eidinow (1923) found that the lethal action of light on infusoria was graded by temperature; they obtained a temperature coefficient for infusoria of nearly 3 between 10° C. and 20° C., these results suggesting that temperature was accelerating a secondary effect of the nature of a chemical reaction, and not a pure photo-chemical action alone, which has a low temperature coefficient—about 1. They reviewed the literature dealing with the influence of temperature upon the action of light.

As already stated, Dreyer and Jansen (1905) showed that ultra-violet rays produced capillary stasis, although they did not determine exactly how such stasis was produced. We have tested the effect of temperature on the action of ultra-violet radiation, using the production of this stasis in the capillaries of the mesentery of the frog as the indicator. Our experiments are given in Table I. We used the unfocussed total radiation from the mercury vapour lamp placed at 12 in. distance from the mesentery, which was immersed in the saline bath. We found, as did Dreyer and Jansen, that stasis was produced

only in the area exposed to the rays. With an average temperature of about 9·5° C. stasis of the capillaries was produced in about 49 minutes (see Exp., Series A, Table I), and that with an average temperature of about 16·5° C. stasis was produced in about 32 minutes (see Exp., Series B, Table I) whilst with a temperature of about 35° C. stasis was produced in about 19 minutes (see Exp., Series C, Table I). Therefore the action of ultra-violet rays from the mercury vapour lamp was graded by temperature: the higher the temperature between the limits tested, 6° and 36° C., the more rapidly was stasis produced. The temperature coefficient between 10° and 20° C. was between 1 and 2—a low coefficient. Experiments (see Series D, Table I) were carried out to prove that a temperature of 36° C. without radiation did not produce stasis; in this case only congestion was produced.

Interference.—Pech (1920) claims that bleaching of raw cotton and production of erythema of the skin are delayed when red or infra-red rays are added to ultra-violet light; these claims have not so far been confirmed.

Leonard Hill and Eidinow (1924) obtained some evidence that the period of exposure to the mercury vapour lamp necessary to immobilize infusoria was prolonged by simultaneous exposure to red rays from a carbon arc. Further observation showed that the addition of red or yellow rays kept the infusoria moving *actively* for a longer time, but did not delay their death.

Hess and Weinstock (1923) found that a glass screen transmitting ultra-violet rays as short as 302 $\mu\mu$ and almost no red or yellow rays protected rats against rickets when exposed for ten minutes' daily radiation, whereas a screen transmitting the same ultra-violet rays and nearly all the red and yellow did not protect rats unless a longer exposure was given. They considered that with a real equality of ultra-violet intensity the red and yellow rays interfered with the action of the ultra-violet rays. Such equality is, however, doubtful.

In our experiments recorded in Table I we observed that (1) the total unfocussed rays from the mercury vapour lamp at 12 in. distance, (2) the same rays plus focussed visible rays from the carbon arc lamp at 40 in. distance, and (3) the first-named rays plus focussed red rays from the carbon arc lamp at 40 in. distance, produced stasis in the capillaries of the frog's mesentery in the average times of 32·4, 32·3 and 30·7 minutes respectively, the average temperatures being 16·5°, 15·7° and 18·3° C. respectively (see Exp., Series B, E, and F, Table I). In these experiments, therefore, the presence of the visible or red rays in great intensity produced no appreciable alteration in the time of exposure necessary to produce stasis by ultra-violet rays, so that we obtained no evidence of interference. The red rays were obtained by passing the rays from the carbon arc lamp through ruby glass about 1·5 mm. thick. Ultra-violet rays were excluded from the visible rays by passing them through a glass focussing lens; the visible and red rays were focussed to form an area of about 1 cm. diameter on the mesentery. The ultra-violet rays were directed on to one side of the mesentery, and the visible or red rays on to the other side.

EXPERIMENTS WITH THE CARBON ARC LAMP.

Influence of temperature.—The carbon arc was placed at 24 in. distance from the frog's mesentery. The rays were focussed by a quartz lens on to

TABLE I.—*Experiments with Quartz Mercury Vapour Lamp at 12 in. Distance from Frog's Mesentery.*

Series.	No. of obs.	Radiation employed.	Temperature of immersion fluid °C.	Time of exposure or to

TABLE I.—*Experiments with Mercury Vapour Lamp at 12 in. Distance from Frog's Mesentery.*

Series.	No. of obs.	Radiation employed.	Temperature of immersion fluid, °C. Limits.	Temperature of immersion fluid, °C. Average.	Result.	Time of exposure or to produce result. Mins. Limits.	Time of exposure or to produce result. Mins. Average.
A	4	Total unfocussed rays from mercury vapour lamp	6·0–12·5	9·5	Stasis	45·0–60·0	49·2
B	7	„ „ „	15·0–17·0	16·5	„	22·0–42·0	32·4
C	7	„ „ „	30·0–36·0	35·0	Stasis and congestion	14·0–25·0	19·0
D	3	*Nil*	34·0–36·0	35·0	Congestion	36·0–60·0	52·0
E	10	Total unfocussed rays from mercury vapour lamp and focussed visible rays from carbon arc*	14·0–17·0	15·7	Stasis	24·0–45·0	32·3
F	6	Total unfocussed rays from mercury vapour lamp and focussed red rays from carbon arc*	17·0–22·5	18·3	„	20·0–45·0	30·7

* Arc at about 40 in. from mesentery. Glass lens of $5\frac{3}{8}$ in. diameter and 10-in. focus was at about 9 in. from mesentery.

TABLE II.—*Experiments with Carbon Arc Lamp at 24 in. Distance from Frog's Mesentery.*

Rays concentrated by a quartz lens of $2\frac{1}{4}$ in. diameter and 3 in. focus.

Series.	No. of obs.	Immersion fluid for mesentery.	Previous treatment of mesentery.	Screens used.	Temperature of immersion fluid, °C. Limits.	Temperature of immersion fluid, °C. Average.	Result.	Time of exposure or to produce result. Mins. Limits.	Time of exposure or to produce result. Mins. Average.
A	4	0·6 % NaCl	*Nil*	*Nil*	10·0–10·5	10·4	Stasis	6·0– 6·5	6·1
B	5	„	„	„	22·5–25·0	23·4	„	3·0– 5·0	3·8
C	5	„	„	3% quinine	17·0–20·7	19·8	*Nil*	51·0–60·0	55·0
D	5	„	„	Egg white	18·5–20·0	19·3	„	40·0–70·0	50·0
E	10	„	Covered with eosin 1 in 1000 for from 15 to 100 minutes	3% quinine	17·7–20·0	19·2	Stasis	4·0–15·0	7·2
F	8	„	Covered with eosin 1 in 10,000 for from 25 to 180 minutes	„	18·7–21·0	19·3	„	5·0–15·0	9·7
G	4	„	Covered with hæmatoporphyrin 1 in 10,000 for from 8 to 15 minutes	„	17·5–19·0	18·0	Stasis and white thrombi	1·5– 6·0	3·0
H	5	„	Covered with hæmatoporphyrin 1 in 10,000 for from 14 to 75 minutes	A. Melanin; B. Quinine	19·5–20·0	19·7	Do.	2·0–24·0	9·4
I	5	Melanin 1 in 10,000 in 0·6 % NaCl	*Nil*	*Nil*	15·0–22·5	18·0	*Nil*	33·0–105·0	54·0

the mesentery, the exact focus being determined by means of the maximum fluorescence of a piece of uranium glass, which gave the focus of the ultra-violet rays. The lens was about 3 in. from the mesentery. The experiments (see Series A and B, Table II) indicated that the effect of the total focussed rays from the carbon arc lamp were graded by temperature, stasis being produced in the capillaries in an average time of 6·1 minutes at 10·4°C. and in about 3·8 minutes at 23·4° C. Here again the coefficient was between 1 and 2, probably indicating a photochemical reaction, as was the case with the mercury vapour lamp. Our colleague, Dr. Brownlee, calculated the coefficient using Arrhenius's formula, and found $\mu = 5900$ for the experiments with the carbon arc lamp and $\mu = 6400$ for the experiments with the mercury vapour lamp; these are low values for μ.

That the ultra-violet rays were alone responsible for the stasis produced was proved by two sets of experiments (see Series C and D, Table II), in which these rays were excluded or decreased in intensity by fluid screens composed of 3 per cent. quinine and pure egg-white respectively. The screen possessed two layers (one back and one front) of quartz, each of 1 mm. thickness and a fluid thickness of 5 mm. The quinine screen prevented and the albumen screen decreased the biologically active ultra-violet rays falling upon the mesentery, as was seen from the absorption bands in their spectograms. With alteration of the intensity of the ultra-violet rays in this way, no capillary stasis was produced in an average time of about 50 minutes (see Series C and D, Table II). Exposure of egg-white to ultra-violet rays caused the formation of a surface film of coagulated material which further reduced the intensity of the ultra-violet rays. An egg-white screen protected infusoria from ultra-violet rays; this confirms our finding with capillaries.

Screening effect of melanin in fine suspension.—In these experiments (see Series I, Table II) the saline bath was filled with melanin in very fine particulate division (strength 1 in 10,000 in 0·6% NaCl), as shown by the ultra microscope.* The frog's mesentery was immersed in this so that there was a layer of the suspension about 1 cm. thick between the mesentery and the carbon arc, which was placed at 24 in. distance from the mesentery. The total focussed rays from the carbon arc failed to produce stasis in an average time of 54 minutes, so that the melanin particles prevented the ultra-violet light from passing through to the mesentery in sufficient intensity. That some ultra-violet rays did penetrate the suspension was shown by the spectogram of the melanin. In this way, *i.e.* by scattering, the melanin granules of pigmented skin reduce the action of ultra-violet rays. These experiments also indicate that melanin has no sensitizing effect.

Sensitization.—Experiments (Series E and F, Table II) were performed in which eosin solution (1 in 1000 to 1 in 10,000 in 0·6% NaCl) was placed upon the mesentery for some time before it was immersed in the saline bath for exposure to the focussed visible rays of the carbon arc at 24 in., distance. The ultra-violet rays were excluded in these experiments by means of a fluid filter of quinine. It will be observed that stasis was produced by visible rays alone in from 7·2 to 9·7 minutes on an average. Thus the eosin had sensitized the capillaries to visible rays. It was sometimes observed that the capillaries

* We are indebted to Mr. A. Webster for the preparation of this suspension.

were blocked by small collections of white blood-cells, adhering to the endothelial wall.

Raab (1903) found that animals sensitized with eosin suffer from œdema and necrosis of the ears on exposure to light. He also (1900) showed that paramœcia were killed by exposure to visible rays after sensitization with eosin. Adler (1919) proved that ultra-violet light acted as a stimulus to plain muscle, and that visible light had no effect unless the muscle was sensitized with some dye such as eosin.

We carried out similar experiments with hæmatoporphyrin, in which the frog's mesentery had been covered with this substance for some time previous to exposure to visible rays alone. In some experiments (see Series G, Table II) a screen of 3 % quinine was used to absorb the ultra-violet rays of the carbon arc; in others (see Series H, Table II) a thin screen 2 mm. thick of melanin in fine suspension was used in addition. It will be seen that in Series G and H stasis was produced in average times of 3 minutes and 9·4 minutes respectively. The strength of hæmatoporphyrin solution used was about 1 in 10,000 in 0·6% NaCl and weak alkali. In addition to capillary stasis a most striking effect was observed. Both arteries and veins of the frog's mesentery contained pale thrombi, mostly of rounded shape and of various sizes: these thrombi appeared to be entirely free from red cells, which could be seen quite plainly passing the pale thrombi. The first evidence of a thrombus was a minute raised pale body on the endothelial wall. This rapidly increased in size to form a round mass attached at one point of its circumference to the endothelial wall. It seemed as if the endothelial wall attracted and held white blood-cells, and these again attracted and held more white cells, until a rounded mass was formed, sometimes large enough to fill the lumen of the vessel. Usually the thrombus was detached from the wall and hurried along by the current of blood. It required about a minute for the formation of a thrombus large enough to fill the lumen of one of the larger vessels of the mesentery. Capillaries appeared to be blocked by small white thrombi, which evidently played some part at least in the production of stasis. All the above details were observed in the living animal. Stained specimens of frog's mesentery exposed in this way confirmed the above findings. The thrombi were composed of leucocytes, lymphocytes being present in greatest number—of course normally lymphocytes are the most numerous leucocytes in frog's blood. There was no evidence of fibrin. Controls kept in the dark showed no formation of thrombi.

Fig. 3, shows a photograph of a microscopic specimen stained with hæmalum and eosin. The thrombi were seen in all vessels; in some cases instead of separate round masses in the vessels the endothelial wall was plastered with leucocytes, confirming the observation that the endothelial cells attracted by some means and held the leucocytes. There were no hæmorrhages, but in places outside the vessels there was evidence of leucocytosis. In some experiments after the formation of the thrombi the frog was kept alive for several hours unexposed to light, and then the mesentery was stained. In these cases the thrombi had disappeared almost entirely, and the frog's blood-vessels had to a great extent recovered their normal appearance. In other experiments in which hæmatoporphyrin (up to 2 c.c. 1

in 10,000) was injected subcutaneously some hours before exposure to light, similar results as regards thrombi formation were obtained.

We used the mesentery of the urethanized mouse to determine whether the same phenomenon could be produced in the mammal. Hæmatoporphyrin solution—(1 in 2000 to 1 in 10,000) in normal saline and weak alkali—was applied locally in some cases, and in others 1 mgrm. of hæmatoporphyrin was injected subcutaneously. It was more difficult to detect the changes in the blood-vessels of the mouse's mesentery because the vessels were normally embedded in fat. Nevertheless, after some minutes' exposure to the focussed

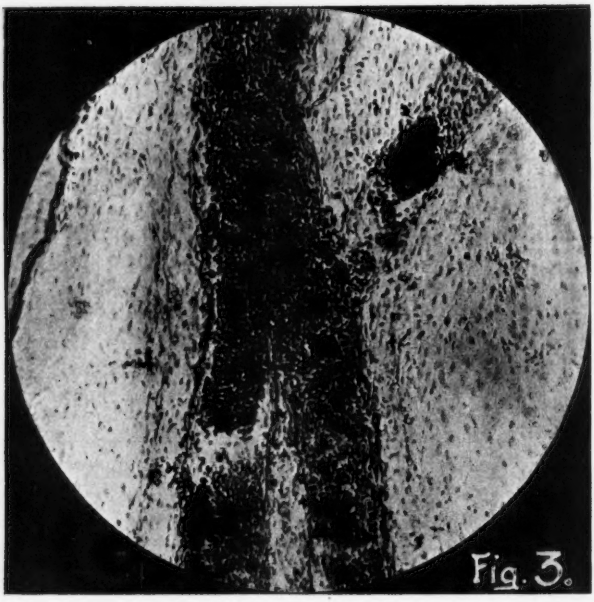


FIG. 3.—Frog's mesentery sensitized to visible rays by hæmatoporphyrin. Thrombi show as dark masses of leucocytes. In one place a prolongation of the thrombus in a venule is seen blocking the terminal portion of a small tributary vessel. The smaller dots in the vessels are chiefly nuclei of red cells. Stained hæmalum and eosin. Scale 120 : 1.

visible rays at 24 in. distance from the carbon arc lamp—the ultra-violet rays being excluded by a quinine filter—pale thrombi were observed in the living animal hurrying along the portions of the smallest arteries uncovered by fat; in some cases these arteries were temporarily blocked by white thrombi, stasis being produced. Some stained specimens showed that capillary blockage by leucocytes had occurred in places; also that leucocytosis into the clear mesentery membrane had been produced in some experiments, after 15 minutes' exposure to light. This leucocytosis into the clear membrane was not observed to any extent in the case of the frog, the changes in this animal

being chiefly inside the blood-vessels. In the case of the mouse the leucocytes appeared to have come sometimes from lymphatic vessels. Leucocytosis was also observed around the blocked capillaries in the edge of the fat surrounding the vessels. Controls showed that such changes did not occur when the mesentery of the sensitized mouse was kept in the dark. The mesentery of the mouse was immersed during exposure to light in a lightly buffered saline solution similar to that used by Evans and Underhill (1923), the temperature being kept at about 37–40° C. The mesentery was not allowed to cool; when the mesentery was not immersed in the saline bath the animal was kept in an electrically heated chamber at about 37° C., the mesentery being kept moist by warm saline.

DISCUSSION.

Many of the above results are of a confirmatory nature. We do not know, however, of any previous experiments in which thrombi, formed of leucocytes alone, have been obtained in the living animal. Bond (1924) refers to a clumping of leucocytes *in vitro*. He states that if a drop of horse-serum be added to a suspension of human leucocytes in normal saline and incubated for a few minutes the leucocytes will tend to run together into clumps, especially in areas around the margin of the cover-glass. He thinks that the tendency for leuco-agglutination to take place on the addition of horse-serum may be associated with the peculiar symptoms which arise when horse-serum is injected into the blood-serum in certain individuals.

Blood-platelets apparently play no great part in the phenomenon we have described above; clumping of blood-platelets is, of course, a well-known phenomenon.

The formation of thrombi of leucocytes observed by us in living animals appears to be a new phenomenon. By sensitization with hæmatoporphyrin the blood-vessels of the mesentery and the leucocytes in the blood therein are obviously markedly affected by visible rays alone. Capillary stasis is produced, just as when ultra-violet rays are used; but ultra-violet rays do not produce these thrombi. Nevertheless, as already pointed out in the introduction to this paper, ultra-violet rays markedly increase the leucocytes—lymphocytes—in the blood of man and animals. We think that, in our sensitization experiments with hæmatoporphyrin, we have obtained perhaps an exaggeration of this normal effect—of ultra-violet light—upon the leucocytes. In most sensitization experiments of any nature the results are the same as those obtained with ultra-violet rays, only that in the sensitization experiments the results are obtained by visible rays, *i. e.* rays of longer wave-length. Hæmatoporphyrin is an artificial, not a natural product; the porphyrins formed in the body are uroporphyrin and stercoporphyrin. Fischer (1916) found that these latter substances are capable of producing sensitization, as does hæmatoporphyrin, only in a less marked degree. It is possible that these substances, stercoporphyrin and uroporphyrin, may be present in the body-cells or blood in very low concentration, and may act under normal conditions in sensitizing the skin to visible rays, at least in so far as the effects upon the leucocytes are concerned. Dr. A. Eidinow has found that the sensitizing of rabbits with small doses of hæmatoporphyrin has no beneficial effect on the hæmo-

bactericidal power when the animals are exposed to light, and the animals subsequently die.

Other effects of radiation upon the skin are more obvious. Thus it has been proved that the ultra-violet rays of about 3000 Angström units are responsible for the erythema and pigmentation of the skin; the rays of this length penetrate about $\frac{1}{10}$ mm. into the epidermis and are absorbed by the cells, at this level, upon which they probably have a marked effect. It is very likely such effect, *e. g.* production of toxic substances, which secondarily produces the well-known pathological effects, *i. e.* exudation diapedesis, dilatation of cutaneous blood-vessels and stasis. Such effects are never produced by visible rays alone when their heating effect is stopped by irrigation. We have shown above that visible rays concentrated from the carbon arc on to the mesentery have no effect in producing capillary stasis when the ultra-violet rays are filtered off by quinine solution, and when the bath is so irrigated to prevent any heating effect. Under similar experimental conditions we have found that no effect was produced upon the skin of the forearm by exposure for 67 minutes to such visible rays alone. Although this is so, we must remember that visible rays penetrate through to the cutaneous blood, which in the absence of any cooling bath they heat up through several degrees Centigrade, as Sonne (1921) first proved, and we confirmed (1923).

SUMMARY.

(1) A simple method for investigation of the biological action of light is described, the determining factor being the production of capillary stasis in the mesentery of the frog.

(2) The action of ultra-violet light in producing capillary stasis may be graded by temperature; the higher the temperature between the limits of 6° and 36° C., the more rapidly is stasis produced. The co-efficient (between 10° and 20° C.) is low—between 1 and 2.

(3) No evidence was obtained that the action of ultra-violet light is interfered with in any way by the presence of visible or red rays in high intensity.

(4) Melanin in very fine suspension acts as an efficient screen to the passage of ultra-violet rays, owing to the scattering of these rays by the fine particles. It has no sensitizing effect.

(5) Eosin (1 in 1000 to 1 in 10,000) applied locally to the frog's mesentery sensitizes the capillaries to visible rays, so that stasis is produced by these rays alone.

(6) Hæmatoporphyrin injected subcutaneously or applied locally to the mesentery (frog or mouse) sensitizes the vessels to visible rays. Capillary stasis is produced and thrombi of leucocytes are formed in the vessels; the thrombi appear at different points along the endothelial wall. Thus further evidence is obtained that light has an important effect upon leucocytes. The endothelial cell of the vessel-wall appears to be involved.

We are indebted to our colleagues, Mr. J. E. Barnard, F.R.S., and Mr. J. Smiles, for help and advice; to Dr. P. P. Laidlaw, who prepared the hæmatoporphyrin used in this research; and to Dr. W. J. Purdy for the diagrams.

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THE DISTRIBUTION OF INSULIN IN HUMAN AND OTHER ANIMAL TISSUES, WITH A DESCRIPTION OF A MICRO-METHOD FOR THE ESTIMATION OF INSULIN IN TISSUES.

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THE history of the isolation and preparation of insulin by Banting and Best (1922) is so well known already that only the very briefest reference need be made to it here.

It will be remembered that the original investigators worked almost exclusively with pancreas, although Collip (1923) recorded the preparation of insulin from blood, and of insulin-like substances from various vegetable tissues. To the latter substances he gave the name "glucokinin," since their pharmacological properties differed from those of insulin. In this country Winter and Smith (1922) published an account of the preparation of insulin from yeast.

Whilst the writers were investigating the insulin content of various organs, several papers appeared on this subject. Thus Best, Smith and Scott

(1923) published an account of the insulin content of the tissues of normal and depancreatized dogs. They state, however, that the combined inaccuracies of the methods of preparation and standardization rob the results of any quantitative value. They record the preparation of active material from organs obtained from depancreatized animals, but their results did not confirm the findings of Ashby (1923), who stated that insulin-like material disappears from the tissues very shortly after pancreatectomy. This observer's preparations raised the blood-sugar at the outset, and acted very similarly to glucokinin. Fisher's (1923) observations threw considerable light on the pharmacological action of insulin-like substances.

He showed that two substances were extracted by the alcohol process, one called the toxic fraction, which raises the blood-sugar, and another which lowers the blood-sugar, and consists of what is generally known as insulin. It would appear that delayed action is due to excess of the toxic fraction, and that the results obtained by many workers were obscured by excess of this fraction.

Since the acetone picrate process, described previously (Dodds and Dickens, 1924[2]), has proved to give much larger yields than the alcohol methods, it was thought that interesting results might be obtained by its application to these problems. After many experiments the following technique was evolved for dealing with small quantities of material. The method about to be described is suitable for quantities of about 10 gm. or even less.

METHOD EMPLOYED.

10 gm. of the tissue are weighed out and minced very finely in some special form of apparatus. We prefer to use the type of mincer used for animal tumours. 0·45 gm. of dry picric acid is added, and the whole is reminced several times until a uniform yellow paste is obtained. If smaller quantities of tissue are employed, an aliquot weight of picric acid is employed. The resulting yellow paste is transferred to a mortar and is ground with 5 to 10 c.c. of absolute acetone. The contents of the mortar are then centrifuged until the supernatant fluid is clear. This is decanted, and the process is repeated twice with 10 c.c. of 70 per cent. acetone and water. The three extracts are poured into an evaporating dish, and the acetone is gently boiled off on a water-bath. Alternatively the acetone may be removed by distillation *in vacuo* at a temperature of about 35°C. When as much acetone as possible has been removed, the precipitated picrate of insulin is separated from the liquid by means of the centrifuge. The supernatant fluid is decanted off, and the precipitate is washed with ether, by centrifuging and decantation. This removes fat and excess of picric acid. The insulin picrate is then converted to the hydrochloride by Dudley and Starling's (1924) process. 2 or 3 c.c. of acid alcohol solution (25 c.c. 3 N.HCl, made up to 100 c.c., with 95 per cent. alcohol) are added to the precipitate in the centrifuge tube, and the contents are stirred with a glass rod. If the fluid is very turbid it can be centrifuged, and the supernatant fluid transferred to another tube by means of a pipette. 10 volumes of absolute acetone are added, and the precipitated hydrochloride is separated by centrifuging. The supernatant acetone is decanted, and the

hydrochloride is washed with ether until all picric acid is removed. The tube is then placed in a sulphuric acid vacuum desiccator until the hydrochloride is quite dry, when it can be weighed. If necessary, the whole operation can be conducted in a weighed centrifuge tube, thus avoiding the necessity for transferring the hydrochloride to another vessel. The whole operation can be carried through in a few hours, and very small quantities of material can be worked up. With care the final product has a rabbit-unit of between 0·25 to 1 mgm.

RESULTS.

By means of this process we have investigated the insulin content of various organs in the ox, pig, horse and sheep. The results are recorded below in tabular form.

Animal.	Organ.	Weight in gm.	Rabbit-unit per gland.	Rabbit-unit per kgm.
Ox	Pancreas	300	750	2500
	Salivary gland	50	35	710
	Ovary	—	—	Inactive
	Liver	3000	600	200
Pig	Pancreas	100	428	4280
	,,	78	294	3000
Horse	,,	250	375	1500
Sheep	,,	100	100	1000
Cat	,,	1	2	2000

Very little idea of the insulin distribution can be gained from these figures owing to the difficulty of obtaining all the organs of one animal. It can be seen, however, that the liver contains almost as much insulin per gland as the pancreas. The figures also show that the pancreas of the horse and sheep are suitable for commercial preparation.

Since the diet of man differs very much from that of animals, it was thought that an investigation into the distribution of insulin throughout the

TABLE A. Rabbit units of insulin in various organs

Age	Sex	Cause of death.	Hrs. between death and analysis	Pancreas		Kidneys		Brain		Spleen		Liver		Muscle	
				per kilo.	per gland	per kilo.	per gland	per kilo.	per organ	per kilo.	per organ.	per kilo.	per organ	per kilo.	per organ
62	Male	Pulmonary embolus	3	870	98·5	1166	365	—	—	900	164	250	410	—	—
60	Female	Sarcoma of neck	6	Nil.	Nil.	—	—	122	122	—	—	40	80	Nil.	Nil.
63	Female	Myocardial degen.	2	1080	43	115	20·7	225	252	—	—	—	—	352	?
32	Male	Basal meningitis	8	Nil.	—	Nil.	—	Nil.	—	Nil.	—	Nil.	—	Nil.	—
Foetus		Stillborn	1	380	5										
43	Female	Diabetic coma	3	425	17	—	—	—	—	—	—	163	180	—	—
44	Female	Acute pancreatitis	8	470	?	40		—	—	—	—	40			

TABLE B. *Vegetable Tissues.*

Plant	Rabbit units per kilo.
Potato	60
Yeast	Nil.
Onion	70

human body would yield interesting results. The difficulties in the way of such an investigation are very great. Firstly the question of freshness of tissues, and secondly the difficulty in procuring normal organs, make the exact estimation problematic.

In Table A will be found the results of analysis performed on cadavers showing various lesions.

COMMENTARY.

The results obtained from the analysis of animal organs are in general agreement with those of other observers, with the exception of Ashby, who stated that many of his non-pancreatic preparations showed the delayed action.

This can be explained by Fisher's work, as already stated. It would appear, from the work of Dudley, that any method involving his purification process would eliminate the toxic fraction, thus accounting for the absence of delayed action in our preparations.

With regard to the analysis of human tissues, some very interesting questions arise. It becomes obvious, from the figures, that the insulin disappears very rapidly after death. Thus in one case, examined after eight hours, no trace of active material could be found in any organ. Also, the insulin content of the organs of a person who has been ill for a long time (*vide* case of meningitis) was considerably lower than that of a person dying of an acute condition (pulmonary embolus). In so far as one can judge from the figures, the pancreas contains very much less insulin per organ than the kidneys, liver, brain and spleen. The insulin content per kilogramme of pancreas, kidneys and spleen seem to be about equal in one case examined within three hours of death. Also, since muscle is responsible for the greater part of the weight of the body, and since it contains 350 rabbit-units per kilogramme, the main store of insulin must be located here.

A possible explanation for the relatively low insulin content of the pancreas when compared to other organs such as kidney and spleen might be found in the fact that the pancreas alone contains a very active proteolyte enzyme. An exaggerated example of this is seen in Case 1. This would tend to destroy the activity immediately death took place.

By far the most striking feature is the fact that the organs of a diabetic dying in coma contain active material in quite large quantities. The pancreas was very small, but gave quite a good yield on the kilogramme basis. The liver also contained a fair quantity. The meaning of these findings is very difficult to see. They correspond with those of Best, Smith and Scott in the depancreatized dog, except that they killed their animals, whilst the patient in our series died from the disease.

In the case of acute pancreatitis only a few grammes of apparently healthy pancreas could be found, the rest being a necrotic, pulpy mass. The yield of insulin from the healthy portion is surprisingly good: one would have expected it to be on the low side, as is the case with the other organs.

The severe toxæmia which exists in these cases may have some effect on the metabolism of the tissues, or it may be that the total output of insulin from the pancreas is small, and the reserve in the tissues called upon. But

on the other hand, the presence of insulin in considerable amount in the tissues of the diabetic suggests that death was not directly due to its absence. This can be explained in one of the following ways:

1. That the death in coma may be due to other retained products circulating in the system owing to changes within the kidney associated with (chronic) diabetes.

2. Or that the absence of insulin may not be the sole factor in the production of fatal diabetic coma.

When further material comes to hand, more light may be thrown on this interesting problem.

Further experiments on the lines given above are in progress, and it is hoped, at a future date, to publish a more exact account of the distribution of insulin throughout the body.

SUMMARY.

(1) The insulin content of some of the organs of the ox, pig, sheep, horse and cat has been worked out.

(2) The insulin content per kilogramme of the kidneys and spleen has been found to be roughly the same as that of the pancreas in the human subject.

(3) The insulin content of the organs of a person dying in diabetic coma was found to be quite appreciable.

(4) No delayed action was found with any of the preparations, whether from the pancreas or elsewhere.

(5) Active material, indistinguishable from insulin, was prepared from potatoes and onions.

The rabbit-unit employed in this investigation was given by the weight of insulin required to lower the blood-sugar of a kilogramme rabbit, previously starved for 24 hours, from the normal level to 0·045 per cent. in a period of 4 hours. All standardizations were performed on at least three rabbits.

Part of the expenses of this investigation was borne by the Medical Research Council in a grant to one of us (E. C. D.).

We have to thank Prof. J. McIntosh for his advice and help throughout this investigation.

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THE REACTION OF THE SKIN TO VACCINIA VIRUS.

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TABLE OF CONTENTS.

	PAGE
Introduction	332
Source of Material and Experimental Methods Employed	334
Naked-eye Response to Intradermal Inoculation of Vaccine Virus	334
Titration of Viricidal Serum by Intradermal Method	336
Histology of Intradermal Response	338
Histology of Response to Scarification	340
Action of Viricidal Serum on the Histological Picture	341
Skin-Response following Intravenous Inoculation of Virus	341
Histology of Lesion Produced by Subcutaneous Inoculation	342
Inoculation of Virus Directly into Spleen	344
Intraperitoneal Inoculation of Virus	345
Vaccinia and Molluscum in the Fowl	345
The Action of Anti-epidermal Serum	346
General Discussion of Experimental Data	347
Conclusions	349
References	349

INTRODUCTION.

THE very obvious predominance of epidermal lesions in the gross pathological anatomy of variola and vaccinia, whether spontaneous or experimentally induced, has for many years been interpreted as indicating a peculiar affinity of these vira for skin and not merely for skin-tissue as a whole, but for that portion of it which is derived from epiblast.

This very general belief is reflected in all the histological teaching of the past sixty years, and a survey of the relevant literature—most of it of very ancient date—clearly indicates that the structure and development of the pock are to be explained as the consequence of some primary implication of the Malpighian layer of the skin. With regard to the exact nature of this reputed attack of the specific virus on the epiblastic elements, there has been a significant lack of precision and uniformity. Doubtless many of the described morphological alterations in Malpighian elements, such as the "reticular" degeneration, the swelling and "ballooning" and the like, are characteristic enough to deserve mention in any complete account of the total developed lesion, but their interpretation as the outcome of a direct attack of virus on epiblast has invariably meant a failure to ignore the possibility of their being purely secondary phenomena in the course of an inflammatory response on the part of the neighbouring mesoblastic elements. It is probable that the neglect

to consider the vascular response in the dermis has been due largely to the use of casual material, and to the failure so to organize experiment as to enable the development of the vaccinial attack to be followed from its earliest manifestations onwards. The search for "parasites" in epiblastic elements has also probably withdrawn attention from dermal lesions. It is true that the histological literature of variola and vaccinia contains incidental references to vascular lesions noted in the dermis at various stages in the development of the pock. In the elaborate study of variola and vaccinia by Councilman, Magrath, Brinckerhoff, Tyzzer, Southard, Thompson, Bancroft and Calkins (1904), to which the reader may be referred for an account of the early literature of this subject dating back to the work of Auspitz and Basch in 1863, it would appear that so long ago as 1879 Cornil drew attention to vascular changes in corium involving particularly the capillary endothelium.

The American workers, in their study of human variolous lesions and of experimental vaccinial lesions in cornea and skin of calves and rabbits, mention the occurrence of corium involvement with perivascular cellular infiltration in certain skin regions implicated in the variolous attack, but the chief object of their labours was to demonstrate, like their predecessors (Weigert, Unna and many others), the primary involvement of epidermis, and the association therewith of an intracellular parasite passing through a complicated developmental cycle. The morphological alterations that may be found in epidermal cells in the course of vaccinia in the calf are very fully detailed by Mann in his joint study with Copeman (Copeman and Mann, 1899).

The belief that variola and vaccinia virus had special affinity for epidermis was fostered also by data derived from immunological experience. Immunity to the vira in question was generally held to reside in the skin itself, and it is surprising that the demonstration by Sternberg (1892) of the fact that the serum of vaccinated animals acquired the property of neutralizing the specific virus *in vitro* so that a mixture of the two failed to take in a fresh animal, did so little to alter general opinion in the direction of postulating a general type of immunity, and that, too, although the presence of these viricidins or neutralizing bodies received ample confirmation from the work of Béclère, Chambon and Menard (1899) and many others.

It may be mentioned here that it was in the course of a study of these viricidins that the present writer was led to a renewed consideration of the histology of experimental vaccinia and to the conclusions enunciated in this communication.

The more recent work showing that in experimental vaccinia a potent virus may be recovered from various organs (Gins, 1916; Gins and Weber, 1916; Otawara, 1921; Watanabe, 1924) has more firmly impressed the view that in variola and vaccinia we have to deal with a virus which, though finding in the skin a seat of predilection for exercising its proliferative activity, is yet distributed generally throughout the body, and is capable of inducing the formation of specific antibodies. Since Sternberg's demonstration in vaccinia we have learned that a general immunity of a similar order occurs after recovery from other ultra-microscopic vira. Nevertheless, in spite of accumulating evidence to the contrary, there has been within the last two years a reaction towards the older view of specific tissue susceptibility and

specific tissue immunity as attributes of the vira in question. Levaditi and Nicolau (1923) on the one hand and Besredka and co-workers on the other have made what are essentially similar claims. The former writers conclude from their experiments that the vaccinia virus possesses a peculiar and fixed affinity for epiblast wherever present. Their work I shall discuss later only in so far as it affects the general question whether epiblast is or is not the essential focus of vaccinial attack. Even if it can be shown, as I propose in this communication, that that attack is characterized by a primary and throughout dominant involvement of the reticulo-endothelial system that may or may not be accompanied by secondary disturbance of adjoining epiblast, there will still remain to be explained why in certain regions the dermal portion of the skin offers the best facilities for the pullulation of the virus. On this point explanations will be offered at a later stage.

SOURCE OF MATERIAL AND EXPERIMENTAL METHODS EMPLOYED.

Vaccine virus.—Regular supplies of material freshly scraped were received from the Institute's lymph laboratories. For use this material (generally about 1 gm.) was ground in a mortar with sterile sand. Normal saline was gradually added and made up to 15 c.c. volume. The sand was allowed to settle, and the emulsion was then centrifugalized at high speed for 10 minutes. The resulting opalescent fluid was usually digested with normal rabbit serum in the cold (equal parts) and again centrifugalized at high speed. This latter refinement greatly facilitated the purification of the lymph from contaminating cocci. Dilutions for inoculation were then made from this fresh material so prepared, or from the same material after storage in the cold with equal parts of glycerine.

Experimental animal.—The rabbit was used throughout.

Methods of inoculation.—(1) Intracutaneous: On white or pink skins depilated on the previous day with freshly prepared mixture of barium and calcium sulphides. (2) Cutaneous: The material being rubbed in with a glass rod over the freshly shaved or lightly scarified skin. (3) Intravenous: In ear vein. (4) Subcutaneous. (5) Intraperitoneal and intrasplenic. (For details of (4) and (5) see appropriate sections.)

Viricidal serum.—This was obtained from rabbits hyperimmunized either by the subcutaneous or intravenous route with emulsions of vaccine virus.

Material for histological study.—See appropriate sections.

GENERAL DESCRIPTION OF THE NAKED-EYE RESPONSE TO INTRADERMALLY INOCULATED VACCINIA.

The intradermal method of introducing vaccine lymph was first studied by Novotný and Schick (1910), on the lines of intradermal tuberculin administration, and while its main use has been for allergic tests, it has been employed with success in primary vaccinations in children (*e.g.* Frankenstein, 1922). For experimental work with the vaccine virus the intradermal route presents certain important advantages, chief of which being that similar or graded doses can be introduced on a series of spots on the same animal, and the resultant reactions compared in some degree quantitatively by the extent of the

inflammatory reaction. Also, as has been done throughout in this work, convenient controls can be made with virus neutralized by viricidal serum. To explain the course of the reaction from the naked-eye standpoint I append the following illustrative experiments: (1) with similar doses on the same rabbit, (2) with graded doses on the same rabbit, (3) with arbitrary dose of virus digested with graduated doses of viricidal serum, and (4) with minimal test dose of virus digested with falling dilutions of immune serum (quantitative titration).

Exp. 1.—Lymph prepared as described above and which had been finally centrifugalized after digestion with normal rabbit serum, was diluted 1 in 10 for use. A set of 10 circles was drawn with a wet copying pencil on a rabbit's back depilated 24 hours previously, each circle being about 1 inch in diameter. In each circle 0·2 c.c. was injected intradermally, the needle being introduced near the edge and directed towards the centre.

The appearance of the circles at various times thereafter are here recorded:

6 *hours.*—No naked-eye change.

24 *hours.*—All circles show a fine pink blush but no obvious thickening or swelling.

48 *hours.*—All circles now very definitely swollen and œdematous and their surfaces red.

3 *days.*—Redness more intense and circles feel harder. Slight but definite scaling on surface.

4 *days.*—Redness less marked but superficial scaling more extensive.

6 *days.*—No further advance except in the direction of scaling, which is very marked. Variable degree of thickening.

7 *days.*—Circles now almost flat and redness almost gone, but still considerable scaling. Spot taken between the fingers still shows definite swelling.

10 *days.*—Circles flat and pale and covered with scales.

15 *days.*—Scaling rapidly disappearing.

The height of the intracutaneous response is usually reached about the fourth day in so far as naked-eye changes are concerned. After that period retrogression begins and proceeds very slowly. As we shall see, however, histological examination of excised areas at these later periods shows an extremely gradual return to the normal histological picture.

Variations from above picture.—Three may be mentioned: (1) With doses of more concentrated material the general course is as above, but the amount and intensity of the inflammatory response in dermis may be such that in place of the ordinary dry scaly exudate overlying an apparently intact epidermis, an actual loss of epidermal substance may occur in the centre. This is plugged with a thick scab under which healing takes place. As is well known, the porosity of the corneal layer in the shaved or depilated rabbit's skin allows the fluid portion of the exudate a ready passage to the exterior, leaving only the cellular elements behind to form the scab. True vesicles rarely occur. (2) Occasionally if care is not taken to get rid of excess of cocci a frank pustule may appear in the centre of the circle even in 24 hours, at a time therefore when the response to the virus is not apparent. Control experiments with staphylococcal emulsions introduced intradermally showed

invariably this rapid development of dermal pustule, easily recognized by the naked eye, and readily distinguishable from the true virus response. (3) With very dilute virus the first response is simply delayed by about 24 hours.

Exp. 2.—*Response to graded doses on the same animal.*

The result may be recorded thus, the + signs indicating intensity and size of resulting inflammatory reaction.

	24 hours.	48 hours.	3 days.	6 days.
Undil.	++	+++	+++	+++
1 in 10	+	+	++	++++
1 in 100	0	Tr.	++	++
1 in 1000	0	Tr.	++	+

The end-point was therefore not reached, and further dilutions of the same fluid were inoculated on a second rabbit with result as follows:

1 in 1000	Tr.	+	++	+++
1 in 10,000	0	Tr.	Tr.	+
1 in 100,000	0	0	0	0
1 in 1,000,000	0	0	0	0

It is clear therefore that in dilutions beyond 1 in 10,000, the lymph may be regarded as incapable of producing a visible response. It will be noticed also that the lower dilutions develop more gradually, becoming definite responses only about the third or fourth day.

TITRATION OF VIRICIDAL SERUM BY INTRADERMAL METHOD.

Exp. 3.—*To illustrate the titration of viricidal sera by the intradermal method, an arbitrary dose of virus being used.*

Three sera were compared, viz. "A," from rabbit on fifth day after scarification, "B," from rabbit on fifteenth day after scarification, and "C," from a hyperimmunized animal.

To each dilution of serum an equal volume of a lymph dilution was added. The control was the same lymph dilution mixed with an equal part of normal rabbit serum. The result was as follows:

	3 days.	5 days.	6 days.
Normal serum + lymph	++	+++	+++++
"A" + ditto	+	++	++
"A" (1 in 10) + ditto	++	+++	++++
"B" + ditto	0	+	+
"B" (1 in 10) + ditto	0	+	++
"B" (1 in 100) + ditto	+	++	+++
"C" + ditto	0	Tr.	0
"C" (1 in 10) + ditto	0	+	+
"C" (1 in 100) + ditto	++	+++	++++
"C" (1 in 1000) + ditto	++	+++	+++++

This animal was killed on the sixth day, in order to secure specimens for histological examination, and at the same time opportunity was taken to control the readings by examination with transmitted light of the portion of skin containing the various spots after reflection from underlying muscle. The

areas of inflammatory response in the various spots stand out very clearly and a rough measurement with calipers is possible. One may note also the small hæmorrhages or hæmorrhagic necroses in the track of the vessels passing through those areas where the inflammatory response has been most marked and their complete absence elsewhere. A cross-section also reveals the extent of the thickening and œdema of dermis. In the above experiment the calipers' record was as follows:

Control . 18	Control . 18	Control . 18
"A" (10) . 10	"B" (100) . 8	"C" (1000) . 15
"A" (pure) . 5	"B" (10) . 3	"C" (100) . 12
	"B" (pure) . 2	"C" (10) . 3
		"C" (pure) . <1

The following experiment is also appended, in which undiluted immune serum and undiluted normal serum was added in equal volumes to falling doses of lymph, and it will be seen that while the minimal reacting dose of lymph was not reached, the neutralizing action of the immune serum stands out clearly.

Dilution of lymph in mixture.	Lymph + saline.	Lymph + normal rabbit serum.	Lymph + immune serum.
1 in 40	+++	+++	+
1 in 80	+++	+++	±
1 in 160	++++	+++	Tr.
1 in 320	++++	+	0
1 in 640	+	+	0
1 in 1280	++	++	0

Exp. 4.—*Outline of a quantitative titrations.**

In Exp. 2 it was determined that the minimal reacting dose of lymph was 1 in 10,000. For use 10 times this dose was taken (1 in 1000), and dilutions of a viricidal serum were mixed with this dose of virus and inoculated intradermally. I append the reading on the 6th day:

Serum (pure) + lymph	*Nil.*
Ditto (1 in 10) + ditto	*Nil.*
Ditto (1 in 100) + ditto	±
Ditto (1 in 1000) + ditto	++
Normal rabbit serum + ditto	+++

This immune serum had consequently a titre of 100 but not 1000. I have thought it necessary to cite these latter experiments, as it was these which suggested the desirability of inquiring into the histological picture in completely neutralized and partially neutralized intradermal reactions. It was

* A method of titrating viricidal serum similar in principle to that here outlined is described by Otawara (1921). From the English abstract of his paper it would appear that the author found the minimal reacting dose of passed testicular virus which produced papules when introduced *under* the rabbit's skin. Having found this dose he took various dilutions of the viricidal serum and mixed each with five times the minimal reacting dose of virus. The mixtures were then inoculated *under* the skin of the rabbit. The titre of the serum was the highest dilution which completely neutralized this dose.

ab initio apparent that the main action of the immune serum in so far as the visible response was concerned lay in abolishing either wholly or partially the redness and œdema. This action alone suggested that the main attack was most probably concentrated on the reticulo-endothelial system in the dermis.

Anti-sheep serum (precipitating serum) was never found to possess any virus-neutralizing action, although this, like the viricidal serum, exerted an energetic agglutinating action on opalescent emulsions of virus. (*The antivariolous lymph used was obtained from the sheep.*)

HISTOLOGY OF THE INTRADERMAL RESPONSE.

The histological investigation of the intradermal response has necessitated the removal of many Schick areas, either taken and fixed at once after killing the animal or removed surgically under an anæsthetic—the latter procedure enabling one to examine from day to day responses to similar volumes of the same virus concentration on the same animal. I propose to take the data from an experiment of this latter kind as a basis for a general summary of the histological picture. In all cases a central segment from the spot was cut with a safety-razor blade and immediately placed in formalin-sublimate-iodine. The stains used were principally Twort's light-green-neutral-red mixture* and Dominici's stain. The former gave most excellent differential staining of the various skin elements.

Removal at 6 hours.—Epidermal structures: Cells of Malpighian layer intact and well stained. Very slight œdema of cytoplasm with shrinkage of nucleus to centre of cell. No penetration of Malpighian layer by polynuclears. *Hair-follicles:* Nuclei intact. Polynuclears infiltrating between the cells. These polynuclears are intact and readily recognizable by their acidophile granules. *Dermal structures:* Venous channels congested and many packed with polynuclears. Infiltration of dermis (except papillary region) with polynuclears. Slight œdema in tissue spaces.

Removal at 24 hours.—Epidermal structures: Malpighian cells intact and well stained. Some œdema and dislocation of nuclei only in the deep germinative layer consequent on œdema in the interpapillary region. Polynuclears which have infiltrated hair-follicles now show signs of karyorrhexis. *Dermal structures:* Infiltrating polynuclears now greatly increased and on the whole appear healthy. Minute capillaries in papillary region and overlying skin muscle present swollen or frayed-out endothelia and occasional mitoses are seen (Fig. 4). Endothelioid cells from vessel-sheaths (adventitial cells of Marchand or hæmohistioblasts of Ferrata) have commenced to engulf effete polynuclears. (The term "adventitial cell" will be used in all that follows, though their histogenetic relationships, on the one hand with the capillary endothelium, and on the other with the connective-tissue cell as elaborated in Ferrata's scheme (1918) of the reticulo-endothelial system, are freely accepted.)

Removal at 2 days.—Epidermal structures: Malpighian cells still little affected but show some infiltration with polynuclears. Cells of hair-follicles show some œdema, and polynuclears which have penetrated to their vicinity show rhectic changes giving the appearance of "inclusions." Even when rhexis of polynuclear chromatin has advanced to extreme comminution, the origin of the particles can usually be detected with ease by the fact that the acidophile granules (green with Twort's stain) persist in their vicinity. *Dermal structures:* The dermis now shows a commencing separation into three layers. A central area contains karyorrhectic polynuclears and necrosed or hyaline tissue fibres, and occasional large vessels whose walls right up to the endothelium are infiltrated with polynuclears, some of the latter being included in the endothelium. Fibrin threads are also visible in the vicinity of these vessels. This central "battle-ground" (Fig. 14) is bounded above and below by dense

* A full description of this stain appears for the first time in a recent paper by F. W. Twort (1924), *J. State Med.*, **32**, 351. (A note on an improved technique appears in the present issue of this Journal.—Ed.)

infiltrates of polynuclears, adventitial cells devouring them, and small vessels from whose sheaths adventitial cells are being actively produced and thrown off. Adventitial cells are also proliferating in sheaths of vessels overlying skin muscle, and this same process is extending to the perimysial capillaries (Fig. 1). Some vessels in central zone show complete coagulation necrosis of their walls with exuded fibrin in the neighbourhood.

Removal after 3 *days.—Epidermal structures:* In certain areas only, cells of Malpighian layer and of hair-follicles show very definite chromatolysis and swelling, but not quite sufficient to justify the term "ballooning." Even in such swollen cells mitoses are frequently seen. Between and in the cells are remains of karyorrhectic polynuclears reduced to remarkably small and uniformly round or oval chromatin particles. Here and there lying between the corneum and the upper portion of the Malpighian layer are lens-shaped collections of closely packed effete cells, which have come from the depth to form the scabs or scales visible to the naked eye. Owing to this advancing exudate the bottom portions of the follicles are becoming disorganized. *Dermal structures:* Central zone is now very clearly marked, and consists of finely comminuted polynuclear chromatin and isolated necrosed vessels, the latter often presenting extensive hæmorrhages in the vicinity. Proliferation of adventitial cells is proceeding actively over skin muscle and throughout perimysium (Figs. 11 and 12).

Removal at 4 *days.—Epidermal structures:* Ballooning of Malpighian cells and some chromatolysis are now evident. There is, however, good apposition, and mitoses are frequently seen even in ballooned cells. Deep portions of hair-follicles have in many cases disappeared, and their places taken by young growing vascular tissue with many mitotic adventitial cells. The Malpighian layer is not so far disorganized. *Dermal structures:* Central zone remains as before. In the boundary zones, infiltrating polynuclears are receding and new vascular tissue is being actively formed.

Removal at 7 *days.—Epidermal structures:* Malpighian cells have a rather shrunken appearance, suggesting recovery from the œdematous condition. Mitoses are numerous. *Dermal structures:* Central zone is still present, but is being encroached upon by the proliferating vascular tissue in the boundary zones. The adventitial cells are now in many cases arranged in sleeves round the vessels. Mitoses are common not only in adventitial cells, but also in capillary endothelia. Occasionally one notes for the first time a tranformation of adventitial cells to the plasma-cell type.

Removal at 10 *days.—Epidermal structures:* Malpighian layers now appear quite healthy, and apart from the destruction of the deep portions of hair-follicles by the advancing exudate and the proliferating adventitial cells from the vessels of the follicular sheaths, there is no noticeable implication of epiblastic elements. *Dermal structures:* Central zone is rapidly diminishing in breadth, and already young connective-tissue fibres are being laid down from the new reticular tissue at its confines. The vessels on each side of the central zone possess wide sleeves.

Removal at 15 *days.*—Regeneration of follicles is now proceeding. Central zone of dermis is now gone and its place taken by young fibres and histiocytes. It would appear that many of the adventitial cells produced in excess of requirements disappear by chromatolysis, while others become directly transformed into young connective-tissue cells by a process of lengthening and tapering of the cytoplasm on each side of the nucleus. There is a suggestion that transformation to the plasma-cell type is a step in the transition from adventitial cell to histiocyte, the deeply basophile cytoplasm of the plasma-cell going to form the young collagenous material of the fibre. Over the skin muscle and in the papillary region vessels with dense sleeves are still seen, but apart from this the histological picture is almost restored to normal.

Later spots were not examined from this animal, but it may be mentioned that in another rabbit a spot removed at 17 days still showed diffuse infiltration of the dermis with adventitial cells (Fig. 8)—a picture recalling exactly that met with in the later stages of absorption of diphtheria bacilli introduced intradermally, *e. g.* in the naturally immune rat (unpublished observations).

The above description of the intradermal response represents the essential sequence of changes following a moderate dose of the virus. More concentrated doses produce simply an intensification of these inflammatory changes, which tend, however, to implicate the epidermal elements still more severely. For example, the vascular response in the shape of polynuclear

infiltration and œdema may disorganize completely the hair-follicles, and even break directly through the Malpighian layer, with consequent denudation, which has to be made good by new epidermis advancing from the periphery.

HISTOLOGY OF THE RESPONSE TO SCARIFICATION.

The very decided implication of the reticulo-endothelial system following intradermal application of the virus and the apparently purely secondary implication of the epidermis necessitated a renewed study of the changes following ordinary scarification. As with the intradermal sets, circles were drawn, and within each circle a similar volume of virus was rubbed in with a glass rod. Circles were removed at stated intervals. It will be unnecessary to give the histological pictures in detail, as the sequence of events presented no material difference from that following intradermal inoculation.

Removal at 24 *hours.*—A few plano-convex scabs are already present, though it is possible that the irritation following the epilation with sulphide may have given rise to some (Figs. 13 and 15). No change in Malpighian or follicular cells. In the dermis the capillaries are widely open and full of red cells. Endothelia of capillaries are frayed out. Many polynuclears have invaded the interpapillary region, but in the deeper parts of dermis there is little obvious except dilatation of capillaries.

Removal at 3 *days.*—The Malpighian layer is in part destroyed by exudate advancing from below and the follicles are densely infiltrated with polynuclears. Some small areas of fibrinous œdema are present in the Malpighian layer (abortive vesicle formation) with ballooning and karyorrhexis of neighbouring cells. Dermis is densely infiltrated with polynuclears, especially in the interpapillary region and at bases of hair-follicles. Adventitial cells are numerous and in active mitosis.

Removal at 5 *days.*—A thick scab has now formed over the central denuded portion of epidermis. In the dermis œdema is still more intense and vessels are highly congested. The area of primary conflict or "battle-ground," consisting of necrosed and hyaline fibres and comminuted polynuclear chromatin, is situated in the papillary zones, and is more diffusely distributed than in the intradermal picture, as might indeed be expected. In the deeper parts of dermis the vascular tissue surrounding the disorganized hair-follicles shows active proliferation of adventitial cells—a development which is ultimately to bring about the replacement of dead or atrophied follicular epidermis by embryonic reticulo-endothelial tissue. The larger vessels overlying the skin muscle have now dense sheaths (Figs. 7 and 9).

Removal at 7 *days and* 9 *days.*—The diffuse infiltration of dermis with adventitial cells and the perivascular sheathing have still further progressed. The constituent adventitial cells forming the diffuse infiltrates assume a reticular structure, recalling that of a myxoma by virtue of their intercommunicating cytoplasmic processes. The clumps of dead epidermal cells belonging to disorganized follicles are simply permeated and replaced by this newly-formed vascular tissue. The condition is in fact strictly analogous to the so-called neuronophagic pictures met with in the poliomyelitic cord (Fig. 5).

Removal at 12 *days.*—The scab over the denuded epidermis has fallen off, showing new epidermis below. While the deep portions of dermis are now clear the upper papillary regions are still beset with embryonic vascular tissue, from which, however, new fibres are beginning to be laid down. Among the constituent adventitial cells *multinucleate or giant forms* are occasionally found. They are similar to those met with in the infiltrates following subcutaneous inoculation of the virus (see later).

Removal at 19 *days and* 27 *days.*—Specimens at these dates show a very gradual return to normal conditions, but even at 27 days the upper papillary regions are not yet free of reticular tissue. At these late periods the still remaining embryonic vascular tissue appears to perform the useful function of permeating and snaring off exuberant epidermal processes which have formed under scab.

Summary of cutaneous response (*scarification*).—The earliest change is represented by attack on the capillary system in the papillary region, and to this area of dermis including that in approximation to the hair-follicles the

lesion is confined, deep portions of dermis being only slightly affected. Epiblastic elements of Malpighian layer and hair-follicles involved in the vaccinial lesion are seriously disorganized by the cellular and fluid exudate coming from the inflammatory area immediately below them. They are more intimately involved here than in the intradermal lesions, as one might expect on proximity grounds alone. Serious implication of Malpighian layer and hair-follicles can, however, be avoided almost entirely by reducing the dose of virus. The diffuse and perivascular infiltrates remain the dominant features of the response precisely as in the intradermal reaction.

THE ACTION OF VIRICIDAL SERUM ON THE HISTOLOGICAL PICTURE.

Spots in which no naked-eye change whatever has taken place owing to complete neutralization by viricidal serum have been removed for study. They have shown, when histologically examined, no disturbance of skin-elements apart from banal alterations that may follow the introduction of any foreign substance such as the sheep constituents of the virus or even the rabbit serum itself. Irritation of capillary endothelium and œdema are in complete abeyance. It is of interest also that the neutralization of virus that is unduly charged with contaminating cocci stultifies any action that may be exerted by the cocci themselves. It would seem that these cocci flourish only in the œdema fluid succeeding the virus attack, and that if the virus is rendered impotent the cocci play little or no part. There is no reason to suppose that the viricidal serum possesses any marked bactericidal action on skin cocci contaminating the virus. This was supposed to be the case by Camus (1908), but he found later that any action in this respect possessed by viricidal serum was equally true of normal rabbit serum. It will be noted also later in the histological study of the subcutaneous response that cocci flourished only when the fluid portion of the exudate became confined by a thick pyogenic membrane. In this central fluid cavity cocci flourished abundantly.

Incomplete neutralization of virus.—It has been explained in connection with the experiments illustrating the titration of viricidal sera that small atypical reactions occurred. These took the form of little hardish bumps in the centre of the inoculated spot and accompanied by only a slight degree of redness and œdema. As a rule they would appear late and recede rather rapidly. Histologically these bumps consisted simply of dense infiltrates of adventitial cells, both diffuse and perivascular, especially the latter. These infiltrates surrounded a yet unabsorbed area of necrotic *débris* representing the usual primary battle-ground. In such cases there was no implication of epidermis. The lesion as a whole resembled that met with in unneutralized intradermal reactions in the rabbit's ear (see later) and in the hen's comb. Plasma-cells were noted early in these lesions, indicating a rapid transformation of the embryonic vascular tissue to the normal dermal elements.

SKIN RESPONSE FOLLOWING INTRAVENOUS INOCULATION OF VIRUS.

Repeated attempts were made to bring off the well-known experiment of Calmette and Guérin (1901), who showed that if an intravenous inoculation of

lymph was given to the rabbit and a portion of the skin thereafter shaved, vaccinial papules appeared about the third day on the shaved area. As Gins and Weber (1916) and Watanabe (1924) have also experienced, these attempts were by no means uniformly successful, and, when successful, the skin lesions resulting were not of a dramatic order and very rapidly aborted. In the first place, difficulty was encountered owing to the use of lymph that had not been sufficiently freed from cocci. Such lymph administered intravenously in the unglycerinated state caused the death of the rabbits in two to four days. At autopsy these rabbits showed gross lesions of pyonephritis with large emboli of staphylococci in the vessels of the kidney and elsewhere. Cocci were recovered in pure culture from the heart-blood and kidney. It may be noted that the blood of these animals while suffering from septicæmia following the inoculation of such lymph presented curious changes in the granulation of the polynuclears. With Giemsa staining the cytoplasm was found to contain numerous large ring-shaped or lumpy granules, not acidophile in tint, but taking up rather the polychrome element of the stain. Their appearance at first suggested something of the nature of a parasitic element—perhaps an included virus. Their exact origin was not fully elucidated, but from examination of bone-marrows it was decided that the peculiar granules in the polynuclears represented most probably the remnants of basophile myelocytic granules. A leucocytosis was always present, and many of the polynuclears so affected were immature. With Giemsa staining normal rabbit polynuclears were generally found to contain a few lilac-tinted granules of the ring type in addition to the fully-developed acidophile granules.

The use of glycerinated material for intravenous use gave satisfactory results, and it was possible in this way to secure excellent viricidal serum from animals hyperimmunized by this route.

In two cases a positive Calmette-Guérin result was obtained following a single intravenous dose of virus. In one case two or three low flat reddish papules appeared on the shaved skin on the fourth day. Of these only one became very suspicious, and it was cut out for histological study on the eighth day. In the other successful experiment one minute red papule appeared on the fourth day, but by the sixth day had almost vanished. Though, however, skin lesions on shaved areas were few and unimpressive, small intracutaneous nodules were noted frequently in the rabbit's ear close to the site of the intravenous inoculation, and also occasionally in the opposite ear. These nodules felt like small shot, and were strictly intradermal. Similar nodules were elicited by inoculating virus intradermally in the hairless skin of the inside of the ear, control spots being charged with virus mixed with viricidal serum. The histology of the skin papules and of the ear nodules was similar, the lesion consisting of the usual type of dense inflammatory response in dermis with little or no implication of epidermal elements (Fig. 10).

HISTOLOGY OF LESIONS PRODUCED BY SUBCUTANEOUS INOCULATION.

It has long been known that subcutaneous inoculation of lymph gives effective immunity and may be employed in man. The histology of the lesion thus produced in the subcutis was studied with a view to determining whether

any extension of virus action took place towards the alleged highly susceptible epidermis, and also whether the inflammatory response resembled that met with in the skin. Two such experiments have yielded material for histological study.

Exp. 1.—Glycerinated virus was introduced deeply into the subcutis in the region of the inguinal gland. On the third day a hard thick string of tissue was felt in the neighbourhood of enlarged glands. This hard mass became more obvious during the following three days, and the animal was killed on the seventh day. At the site of inoculation there was considerable hæmorrhage in the subcutis, and spreading yellowish tracts indicating most probably the specific cellular exudate were seen overlying the muscle fascia, and extending in the matted tissue to the dermis about the level of the skin muscle. Sections were made through skin and underlying mass. The epidermis was found to be completely uninvolved. The yellowish tracts seen with the naked eye consisted of comminuted polynuclear *débris* and adventitial cells containing included polynuclears. Surrounding these tracts were great areas of embryonic vascular tissue with numerous newly formed capillaries (Fig. 16). Some of these contained blood while others had extensive areas of hæmorrhage in their vicinity. Between the capillaries were vast numbers of adventitial cells, some of which were multinucleate. It is clear that the specific exudate (corresponding to the scab in the intradermal lesion) was being removed by devious tracks either by way of the neighbouring lymph-glands or towards the exterior. There was no definite fluid cavity as in the second case described below.

Exp. 2.—The virus was in this case inoculated deeply in the abdominal wall. A fluctuating swelling about the size of a walnut resulted, and the animal was killed on the fifth day. The centre was punctured with capillary pipette, and the faintly turbid serous fluid was removed for testing on a fresh rabbit. Rounding the cavity was a tough slough, succeeded by layers of looser material. The overlying skin appeared quite intact.

Histology of a cross-section involving skin and subcutis.—Malpighian layer and hair-follicles appeared healthy. No obvious change was noticeable till the level of the deeper hair-follicles was reached, when diffuse infiltrates of adventitial cells made their appearance along with infiltrating pus-cells. Then came the slough, consisting of necrosed material and clumps of comminuted polynuclear chromatin, which retained their staining properties like the similar masses in the lens scabs following intradermal inoculation. In the centre (fluid removed) was necrotic *débris* with many clumps of cocci. It was here that the leucocidal action of cocci played a part. The polynuclears in this situation had a washed-out appearance, and their nuclei had disappeared. Proceeding deeper the slough was bounded by a zone of new vascular tissue with abundant capillary formation. There were no staphylococci here. Diffuse infiltrations of adventitial cells were present, but no perivascular sheaths. Then came a layer of œdematous tissue with comminuted polynuclear *débris*, and finally a layer of adventitial cells and young newly formed collagenous fibres. The change in the subcutis is essentially similar to that in the dermis, the only difference being that the fluid portion of the exudate collects in the centre, and the cellular portion appears as a

slough structurally parallel to the scab. The reactive changes on the part of the reticulo-endothelial tissue are identical, with the exception that perivascular sheathing is not a prominent feature and is replaced by diffuse infiltrates—a well-recognized variation that may be noted, *e.g.*, in poliomyelitic lesions. That cocci incorporated with the lymph played only a minor part in the total lesion was further clear from the fact that in a second rabbit similarly inoculated with the same virus mixed with an equal quantity of viricidal serum, no swelling resulted. The subcutis presented only a slight thickening not bigger than a pea.

Test for presence of virus in central fluid and in pounded-up pyogenic membrane.—Three equal areas (each about two square inches) on the shaved back of a rabbit were scarified thus: (*a*) With glycerinated sheep lymph; (*b*) with central fluid which had been mixed with equal parts of glycerine; and (*c*) glycerinated slough bordering cavity.

(*a*) Gave a profuse confluent take, (*b*) gave no definite papules, while (*c*) yielded three very large typical papules.

Active virus was therefore present in the wall but not in the fluid contents of the cavity. I do not comment on the finding, but simply note that with other vira, *e. g.* that of foot and mouth disease, similar experiences have been reported. Very probably owing to the early appearance of viricidal bodies in the blood, the potency of virus free in the central fluid may be impaired, while virus which is tucked away in the slough is protected from their action.

INOCULATION OF VIRUS DIRECTLY INTO SPLEEN.

With the object of studying the nature of the primary vaccinial attack when the virus is introduced to a region still more remote from epidermis, glycerinated lymph (0·2 c.c.) was inoculated immediately below the capsule of the spleen after preliminary laparotomy, a white rabbit about 8 weeks old being used. (The experiment was kindly performed for me by Dr. Bedson.)

When the needle was being withdrawn a slight blood spill occurred though the point of entrance was immediately cauterized.

The laparotomy wound healed readily.

On the sixth day the animal was killed. The spleen was quite free from adhesions and was not enlarged. For about a quarter of an inch on each side of the point of inoculation the capsule was thickened and had a dead white appearance, while on the opposite side of the organ a similar thick white area was present and protruded slightly above the level of the spleen surface. These two white areas were found to be continuous in cross-section of the organ. On the adjacent abdominal wall were several raised white protruding nodules, apparently extending deeply into the substance of the abdominal wall. *On the peritoneal coat of the large intestine were numerous minute pin-point white nodules.* Otherwise autopsy showed nothing obvious.

Histology of spleen lesion.—The thick white plaques consisted of a loosely arranged ramifying reticulum of trabecular cells extending deeply inwards from the capsule and sending ramifications throughout the organ. They contained numerous capillaries, mostly empty, but at the margins of the band were full capillaries and isolated hæmorrhages. The white plaques appeared

to be the outermost defensive line of several primary karyorrhectic foci, which were immediately surrounded by actively dividing cells of the primitive stem-cell type. In the outer rows of these latter cells were many plasma-cells.

Peritoneal nodules of intestine.—These minute nodules presented essentially the same structures as the dermal lesions already described. The little nodes were made up of dense perivascular infiltrates surrounding a small primary focus containing necrotic *débris* and representing the initial point of attack and the settlement of the virus (Fig. 2).

Nodules of abdominal wall in neighbourhood of spleen.—These had the structure of subcutaneous nodules elsewhere described. As in the latter, *multinucleate and giant forms* were present among the actively dividing adventitial cells forming the usual infiltrates. There was no involvement of epidermis.

INTRAPERITONEAL INOCULATION OF VIRUS.

Though intraperitoneal inoculation of virus has not been supposed to lead to any obvious gross lesions, it is known to be effective in rendering the animal immune. The finding of specific vaccinial tubercles on the peritoneal coat after intrasplenic inoculation led to the surmise that possibly local lesions due to the virus might be found if carefully searched for. A rabbit was inoculated intraperitoneally with glycerinated virus and killed on the sixth day. The curled-up mesentery showed three or four small yellowish nodules but none such were visible elsewhere. These nodules presented the same histology as the peritoneal tubercles of intestine after intrasplenic inoculation. In the centre of each nodule was a necrotic focus with karyorrhectic *débris* of polynuclear cells. Outside this was a zone of newly formed capillaries with numerous adventitial cells in the vicinity, many in mitosis. Plasma-cells were also numerous.

VACCINIAL LESIONS IN THE HEN'S COMB AND THEIR COMPARISON WITH THOSE DUE TO MOLLUSCUM VIRUS.

Experiments with vaccinia and with molluscum virus have been made on the hen's comb with a view to studying their comparative histology in the light of experience with vaccinia in the rabbit. I do not propose to detail the histology of molluscum lesions, which have been the object of frequent study, though not of recent years. Burnet (1906) and others have attached great importance to the peculiar bodies in the epidermal cells and to their probable parasitic origin. These striking objects still remain unexplained. In the writer's opinion their structure and staining properties suggest simply foci of keratinization within the epidermal cell (Figs. 18 and 19). On the other hand, the vascular lesions underlying the active germinative layer of the thickened and exuberant epidermis are unmistakably analogous to those met with in vaccinia, whether in the rabbit dermis or in the hen's comb (Fig. 17).

Vaccinial nodules in the hen's comb produced by scarification have shown no lesion whatever of epidermis. A papule removed on the sixth day presents simply diffuse adventitial infiltrates and perivascular sheathing of the usual type.

An opportunity of examining palate and tongue lesions (so-called avian diphtheria) was afforded by the development in one hen of very severe

diphtheritic throat lesions about the twentieth day after scarification of the comb with molluscum virus. In this case the comb lesions had been very severe and confluent and showed little tendency to clear up. Owing to symptoms of suffocation the hen was sacrificed on the twenty-fourth day after scarification. Sections made through the thick diphtheritic membranes on tongue and palate and underlying tissues showed the predominantly vascular character of the lesion. The irregular and exuberant Malpighian columns were also displaced and distorted by cellular exudate proceeding from the underlying dermis, isolated islets of Malpighian cells with their contained molluscum bodies being found throughout the inflammatory mass (Fig. 3). I may add that so far as experiments in this connection have gone, fowls which have recovered from molluscum are not immune to vaccinia, and *vice-versâ*.

THE ACTION OF AN ANTI-EPIDERMAL SERUM.

The evidence available from histological study of the intradermal and cutaneous response to vaccinia had in my hands lent no support to the view that that virus had any special affinity for epiblastic tissues, and further inquiry had shown that the same type of lesion involving the reticulo-endothelial system resulted when the virus was introduced to areas remote from epidermis. In these circumstances it seemed desirable to ascertain whether the epiblastic elements of skin could be primarily and specifically interfered with by some toxic agent introduced into the dermis, and it was decided to test the action of a serum prepared by immunizing the goat with rabbit epidermis.

The scraped Malpighian layer of rabbit-skin which had been shaved and carefully cleansed was ground up with sand and glycerinized saline. Material so obtained was inoculated subcutaneously into the goat in successive doses, six in all being given. The material was found to be readily absorbed.

The serum of the immunized goat was tested against washed rabbit red corpuscles (4 per cent. emulsion), and found to agglutinate them completely with cake formation in a dilution of 1 in 32, whereas normal goat serum had only a faint agglutinating action in 1 in 4 dilution. Inoculated intradermally the serum (undiluted) produced in 24 hours in the preliminary test a large red swelling presenting centrally a purple zone of hæmorrhage. Normal goat-serum produced also a pink swelling, but less marked, and with no central hæmorrhagic zone. In three days the swelling produced by the normal goat serum had gone, while that produced by the immune serum had also gone down considerably, though the purple hæmorrhagic zone remained. In five days the central zone presented itself as a pit owing to denuded epidermis. The action of the serum was therefore to produce an early and marked central hæmorrhagic necrosis involving the epidermis. In later intradermal experiments with the same serum, which had been filtered and kept in the cold, the intensity of the reaction in the direction of hæmorrhagic necrosis effects was much less marked, but the reaction was sufficiently intense to make the study of its histology at different periods desirable. This was done in the usual way by removing surgically from the same rabbit, at different intervals, spots inoculated with the same dose of immune serum intradermally.

The results may be briefly detailed:

Removal at 6 hours.—Great outpouring of polynuclears and œdema of dermis. Malpighian cells show some œdema and shrinkage of nucleus to centre.

Removal at 24 hours.—Nuclei of Malpighian cells and of hair-follicles in central portion of inoculated area are shrunken to minute dimensions, while the cytoplasm is ragged and frayed (Fig. 6). Capillary endothelium is destroyed owing to toxic action of serum.

Removal at 48 hours.—Capillary endothelium already showing evidence of reactivity, mitosis being frequent. Cells of Malpighian layer shrunken, but occasionally what appears to be an agonal mitosis can be seen.

Removal at 4 days.—Repair has now set in. Malpighian germinative layer now shows fairly numerous mitoses. There are still, however, many shrunken Malpighian cells which are in process of removal to the exterior. Cells of sebaceous glands are also in active division. New capillary channels are now being hollowed out.

Removal at 7 days.—Repair in Malpighian layer still further advanced, with evidence of exuberant growth.

Removal at 13 days.—Regeneration of hair-follicles proceeding. All exudate now gone.

Anti-epidermal serum, therefore, when introduced into the dermis produced when absorbed an early toxic action on capillaries and on the Malpighian elements nourished thereby. The effect was not of long duration and repair was in full swing from the fourth day onwards.

Intravenous inoculation of vaccinia and of anti-epidermal serum simultaneously.—It was surmised that if vaccinia had a specific affinity for epiblast, the simultaneous intravenous introduction of vaccinia and of anti-epidermal serum would favour the settlement of virus in the shaven and prepared skin with papule production. One experiment of this kind was performed, but no local vaccinia appeared.

GENERAL DISCUSSION OF EXPERIMENTAL DATA.

A study of the histology of experimental vaccinial lesions in the rabbit reveals no evidence of any alleged affinity of the virus for epiblastic elements, whether the method of introduction be by scarification or intradermally. Nor is there any evidence of dermotropism (or more strictly epidermotropism) when the virus is introduced into the subcutis or other remote situations such as the peritoneum or spleen. Intravenous inoculation of the virus is not followed by any regular or striking attack on skin which has been prepared by shaving or depilation, and only on two or three occasions out of many trials have definite skin lesions been secured by this method for histological study. My experience in this respect accords with that of Gins and Weber (1916), and of Watanabe (1924). While uncertain in its action, the Calmette-Guérin experiment undoubtedly confirms certain facts of clinical experience pointing to the influence of local irritative stimuli in eliciting skin lesions. On the other hand, the histological study of skin lesions following intravenous inoculation in no way supports the view that epiblast is the focus of attack. These lesions, like those following other methods of introduction of the virus, are, primarily at least, if not solely, lesions of the local reticulo-endothelial system. Epiblast may, indeed, be secondarily involved, or it may entirely escape injury.

Far more constant in fact than lesions on shaved skin following intravenous inoculation are those local dermal tubercles in the rabbit's ear, whether it be the ear into whose vein the virus has been introduced, or the opposite organ.

Such tubercles are essentially the result of reactive response on the part of the local reticulo-endothelial tissue of the local dermis.

There would seem to be little doubt that the dermis, owing to its terminal and non-anastomosing capillary system (see Krogh, 1922), and its confined character, offers opportunities favourable for the pullulation of any virus that may be conveyed to it from the blood- or lymph-stream, and any local vascular disturbance produced by shaving or scarification would but enhance these favourable conditions. Gins and Weber (1916) suggest a similar explanation. Watanabe (1924), in a recently published paper concerned with the distribution of vaccinial virus in the body after intravenous introduction, was able to demonstrate by passage experiment the presence of virus in portions of intact skin removed about the fourth day, and he has reported the occurrence of minute but palpable papules in skin and mucosæ of lips, tongue, and gums. These minute papules were found histologically to represent purely inflammatory subepithelial foci, and epiblast was not involved. This type of exanthem would at least require careful search for its demonstration, and I have not noted it in my work, but I have occasionally observed small papules on the lips in addition to those found in the ear after intravenous inoculation. In Watanabe's view these minute papules were genuine vaccinial lesions, and their histological features recall exactly those met with in the varied material studied in this experimental research. Levaditi and Nicolau (1923), in the course of their experimental evidence supporting, in their view, a peculiar affinity of vaccine virus for epiblast, have studied histologically the response of the epiblastic and mesoblastic elements of skin to the stimulus of epilation. They concluded that proliferative changes in epithelium constituted the main features of the response. When vaccine virus reached such irritated areas as in the Calmette-Guérin experiment, the preliminary diapedesis of polynuclear cells was followed by immediate attack of virus on these previously stimulated epithelial cords, the virus being presumably liberated from diapedesed leucocytes which have taken it up. The "mitotic state" of the epithelial cells favoured the fixation of the virus in them and there it multiplied. Similarly, germinative cells such as those of ovary and testicle were capable of fixing the virus and ensuring its multiplication in an epithelial environment.

Such a view is in my opinion invalidated by the histological evidence here brought forward, which shows that vaccinial lesions represent primarily attacks on reticulo-endothelial tissue, and that epiblastic elements may escape injury throughout the whole course of the vaccinial attack. The presence of occasional mitoses in epithelial cells seems an insecure foundation on which to build a theory of elective affinity as between virus and epiblast. It appears to me far more reasonable to suppose that preliminary irritation opens up capillary channels, and permits the settlement of virus in the confined dermal space, where its further development is secured. With regard to the elective affinity of virus for germinal cells as Levaditi and Nicolau assert, it is well known that the subepithelial spaces in the germinative organs afford opportunity for the pullulation of organisms of very diverse character when introduced elsewhere. The nature of the circulatory conditions in these areas sufficiently explains these focal settlements without postulating any specific attraction of parenchymal cells.

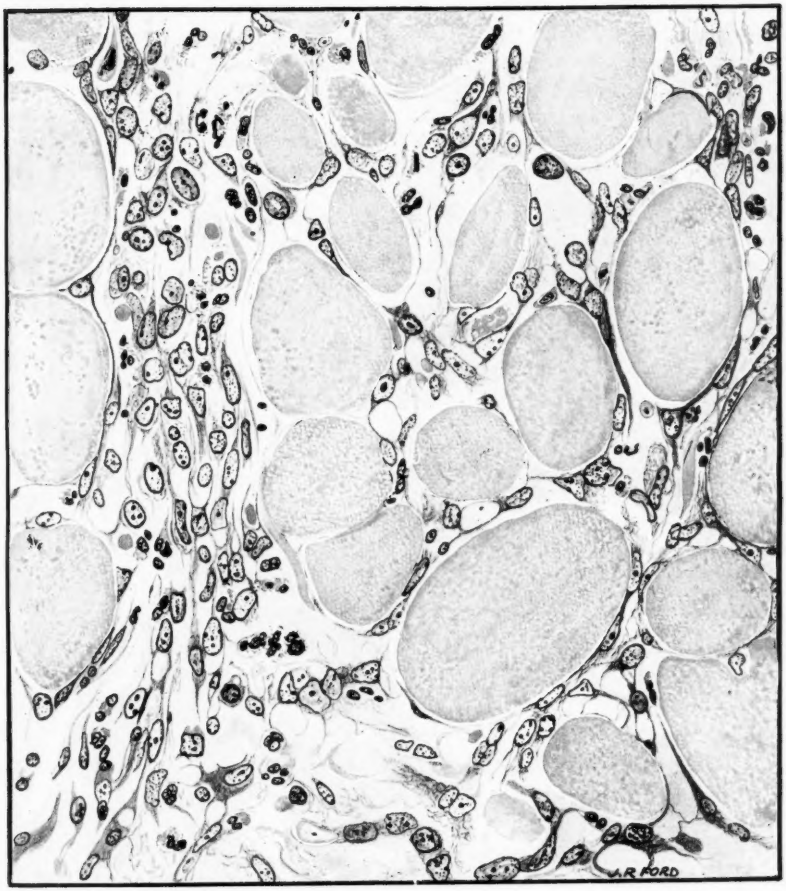

FIG. 1.—Vascular response in perimysium of skin muscle underlying dermis. Intradermal inoculation. Spot removed on the 6th day. Twort's stain.

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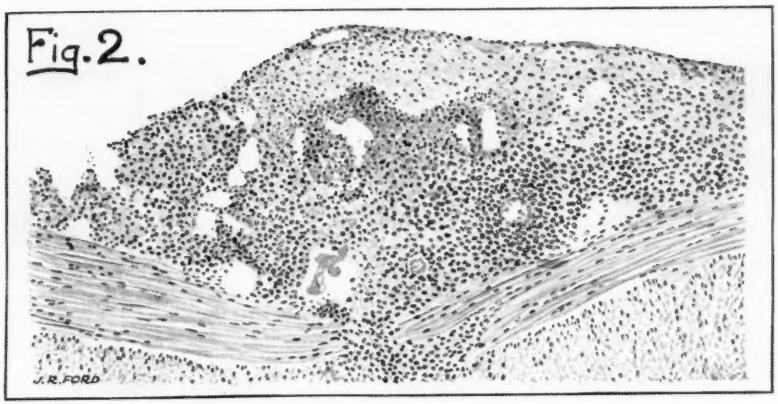

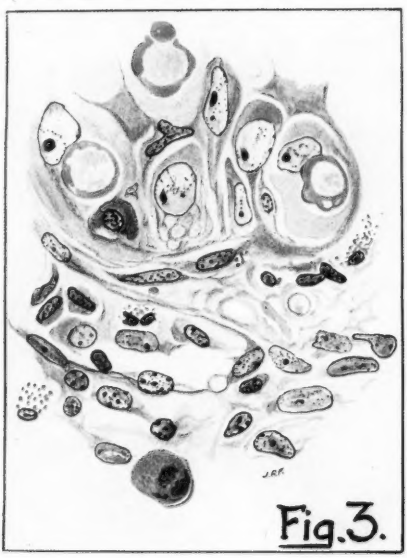

FIG. 2.—Vaccinial nodule on peritoneal coat of intestine. 6th day after inoculation of virus into spleen. Shows primary karyorrhectic focus, diffuse infiltrates and vascular sheathing. Twort's stain.

FIG. 3.—Inflammatory reaction in dermis adjoining germinative layer of epidermis in avian diphtheria. From tongue of fowl on 24th day after scarification of comb with molluscum virus. Molluscum bodies in epithelial cells are stained green. Twort's stain.

FIG. 4.—Capillary in dermis showing mitosis in endothelium with surrounding polymorphonuclears and adventitial cells. Intradermal inoculation. 48 hours. Twort's stain.

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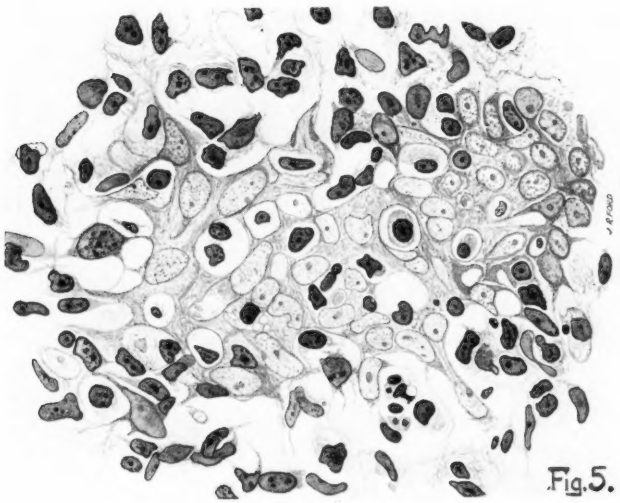

Fig.5.

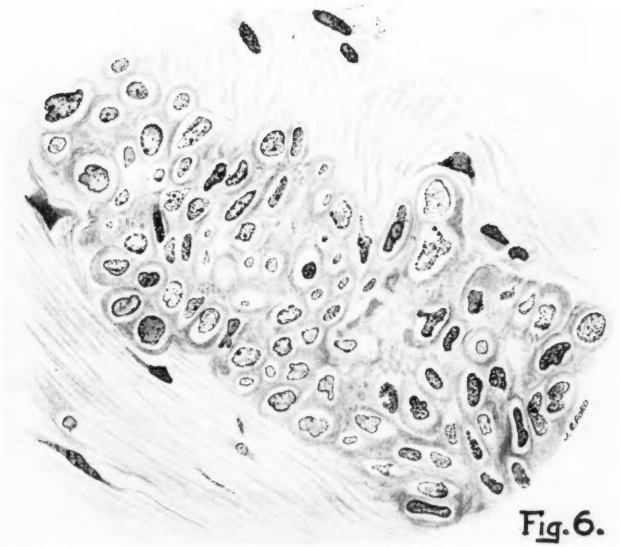

Fig.6.

FIG. 5.—Replacement of dead follicular columns by newly formed reticulo-endothelial tissue. Scarification. 12th day. Twort's stain.

FIG. 6.—Chromatolysis of hair-follicle cells. 24 hours after intradermal inoculation of anti-epidermal serum. Twort's stain.

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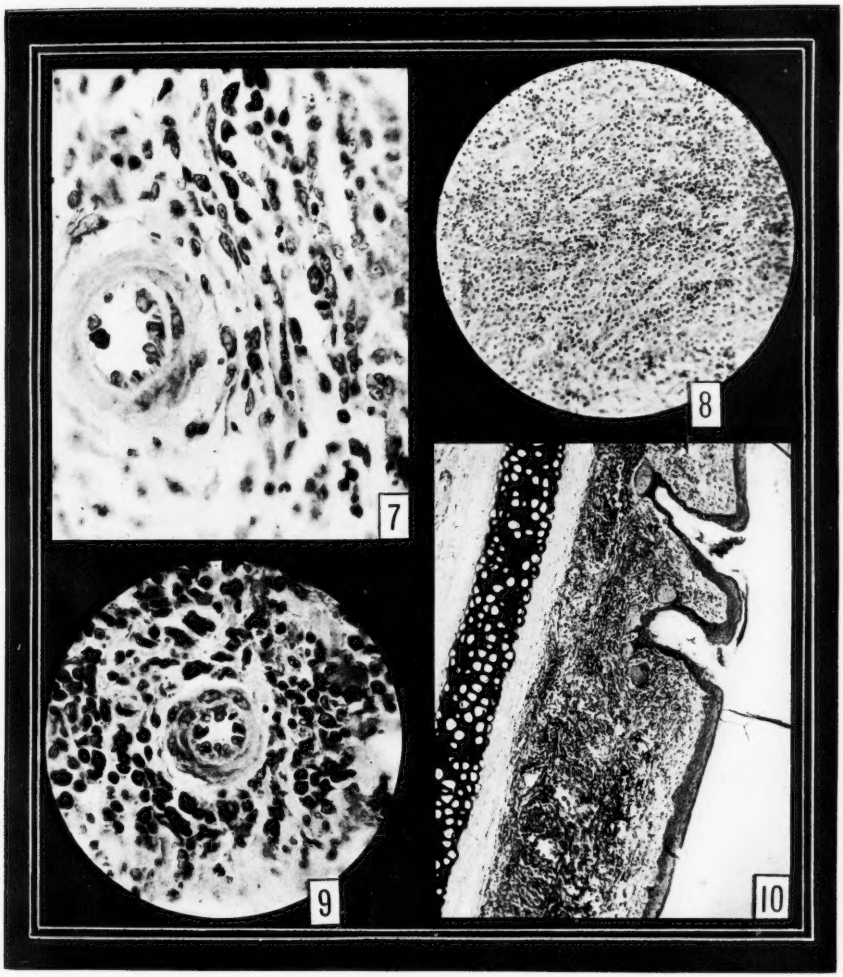

FIG. 7.—Perivascular sheathing in dermis. Scarification. Spot removed on 6th day. Note mitosis in adventitial cell.

FIG. 8.—Diffuse infiltration of dermis with adventitial cells. 17th day after intradermal injection.

FIG. 9.—Perivascular sheathing in dermis. Intradermal inoculation. 6th day.

FIG. 10.—Ear of rabbit. Intradermal inoculation. 8th day. Note the intense vascular reaction in the dermis. No implication of epidermis.

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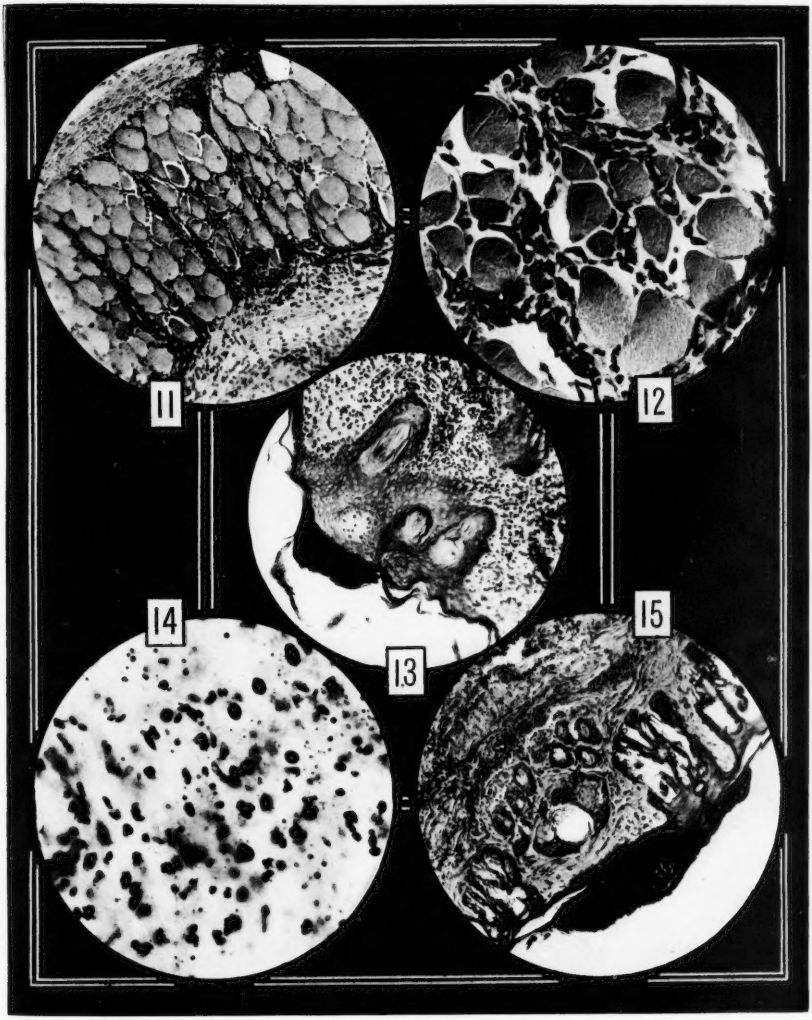

FIG. 11.—Diffuse perivascular infiltrate of adventitial cells in perimysium of skin muscle. It is a direct extension from vascular area overlying skin muscle.

FIG. 12.—Similar to Fig. 11. Scale 120 : 1.

FIG. 13.—Scab between corneum and upper Malpighian layer. Scarification with vaccinia. 7th day.

FIG. 14.—Granular *débris* of polynuclear cells in dermis, showing punctiform comminution of nuclear chromatin. Initial "battle-ground." Intradermal inoculation. 3rd day.

FIG. 15.—Typical lens-shaped scab in usual site between corneum and upper Malpighian layer. Intradermal inoculation of anti-epidermal serum. 6 hours.

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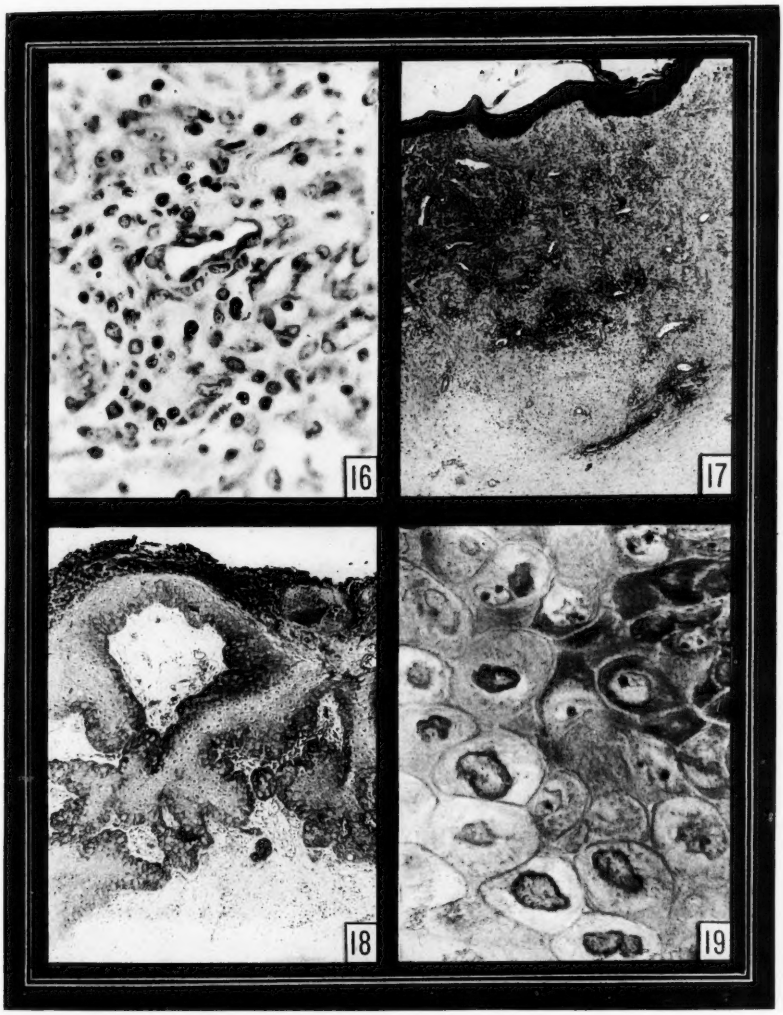

FIG. 16.—Newly formed reticulo-endothelial tissue. Mitosis in capillary endothelium. Subcutaneous inoculation.

FIG. 17.—Vaccinial response in subepithelial tissue of fowl's comb. 13th day. No implication of epidermis.

FIG. 18.—Avian molluscum scarified on comb of fowl. Removal on 10th day. Shows superficial scab overlying epidermis, exuberant epithelial processes with well-marked germinative layer, and molluscum bodies inside epithelial cells.

FIG. 19.—High-power view of epithelial cells from fowl's comb showing the irregular acidophile masses (molluscum bodies) lying outside the nuclei. The latter are excentric and vesicular with one or two darkly staining nucleoli.

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CONCLUSIONS.

(1) The vaccinial lesion has been studied histologically in its various manifestations following cutaneous, intradermal, subcutaneous, intravenous, intraperitoneal and intrasplenic inoculation, and both naked-eye and histological data have been controlled by inoculations of virus mixed with viricidal serum. The lesion is essentially of the order of an acute infective granuloma in which the reticulo-endothelial system is primarily and dominantly involved. There is no evidence that vaccinial virus possesses an elective affinity for epiblastic tissue. The lesions of vaccinia and of molluscum in the hen are histologically of the same order in so far as the dermal response is concerned.

(2) Evidence is afforded that precisely similar tissue responses occur when the virus is introduced into tissues remote from epiblast, such as the subcutis, the peritoneum and the spleen.

(3) In contra-distinction to vaccinia, anti-epidermal serum when introduced intradermally produced an early and definite toxic action on epiblastic elements of skin.

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The photomicrographs (Figs. 7 to 19) were prepared by Dr. D. Reid and Mr. A. Dennis.

FURTHER MODIFICATIONS IN THE PREPARATION OF NEUTRAL RED–LIGHT GREEN DOUBLE STAIN, AND AN IMPROVED METHOD OF EMBEDDING TISSUES IN PARAFFIN.

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In a recent paper (Twort, 1924) on an improved method of preparing the neutral red–light green double stain, it was shown that if the solid dye, prepared by mixing watery solutions of neutral red and light green, is dissolved in 60 per cent. ethyl alcohol in water, in place of pure methyl alcohol, a better staining solution is obtained. In the same paper the technique to be followed for staining under different conditions was also given.

Recent experiments have shown that 60 per cent. normal propyl alcohol in water forms an even better solvent for the dry dye, the solution obtained being quicker and denser in its action. About 1·0 gm. of the solid dye* should be ground up in every 100 c.c. of the solvent, and the solution then filtered. The stock *saturated* solution, when required for use, should be mixed with about an equal quantity of water. Propyl alcohol having a comparatively high boiling-point, there is, when staining, but little evaporation of the alcohol, and therefore but slight tendency to precipitation of the dye. Allyl alcohol may also be used in place of ethyl alcohol, but as sold it may contain some acid, in which case it must be neutralized carefully before use. A 60 per cent. solution of this alcohol in water dissolves more of the dye than any other solvent tested, and the stain so prepared, when mixed with an equal quantity of water, is exceedingly dense and rapid in its action, some spirochætes staining densely in one minute. Normal butyl alcohol may also be used if a saturated solution of the alcohol is made in distilled water, but a saturated solution of the dye in this solvent, which contains less than 10 per cent. of butyl alcohol, will be much weaker than those obtained with the other diluted alcohols already mentioned, and it will be necessary when using this to stain for a longer period. The butyl alcohol staining solution, unlike the others, should be used as such, without the addition of more water. Although not dense, it forms a delicate stain, and it can be recommended for demonstrating fine cellular structure. All the staining solutions stain more densely when used hot.

Acetic acid and pyridin, diluted with water, will also dissolve the dry dye, but the solutions are quite unsuitable for staining purposes, while a solution of the dye in acetone and water is inferior to the staining solutions already described.

If 1 per cent. or more of phenol is added to any of the alcohols used for dissolving the dye, except butyl alcohol, a denser and faster stain results. Its addition to the allyl alcohol stain is recommended when very dense staining is desired.

* Obtainable from G. T. Gurr, 136, New King's Road, London, S.W. 6.

There may be a difficulty with dry film preparations in obtaining well-coloured specimens. Better results, however, are obtained if the films, after being fixed in alcohol, are treated with such solvents as xylol, chloroform, ether, etc., or even better if they are soaked in a solution of soft soap dissolved in 60 per cent. alcohol.

It should be noted particularly that all the rules given in my recent paper for the use of the ethyl alcohol-water stain apply also to the modifications given in the present paper, but the importance of working with an excess of the dye and of getting a saturated solution of this in the particular solvent used may again be emphasized. It is worthy of note that the principles underlying the preparation and use of this stain may in some cases be applied to other double dyes in use by bacteriologists, but the details for these have not yet been worked out. It may also be mentioned that a number of other double dyes have been prepared. These include combinations of neutral red and gentian blue, basic fuchsin and light green, and various combinations of the different basic greens with such acid dyes as naphthalene red, trypanrot, magdala red, orange G., biebrich scarlet, bordeaux red, etc.; but no very satisfactory staining solutions of these have yet been made.

When preparing certain tissues for embedding in paraffin, most workers at times find some difficulty in preventing the tissue from contracting and becoming too hard. This, as is well known, is due partly to the absolute alcohol used in the process of dehydration, but more especially to the xylol, carbon bisulphide, or such similar substance used for clearing the tissue ready for the paraffin bath. Experiments have recently been carried out to see how far it might be possible to substitute these hardening substances in the process of embedding. In the first instance butyl alcohol was tried, as this alcohol mixes well with pure ethyl alcohol and with hot paraffin, and has the additional advantage that it will mix with 50 per cent. ethyl alcohol in water. Experiments showed that the use of butyl alcohol in place of xylol produced very satisfactory results; moreover, the property of this alcohol of mixing with diluted ethyl alcohol made it possible to eliminate absolute ethyl alcohol from the process. Tissues were fixed in formalin-Müller, washed, and placed in dilute ethyl alcohol. They were then placed in 60 per cent. ethyl alcohol, and from this into pure normal butyl alcohol. This was changed several times during the course of two or three hours, and the tissue then passed through several changes of paraffin, in the usual manner. Tissues treated in this manner remain soft and show but little if any shrinkage. Thin sections can be obtained with ease, and these when stained and examined under the microscope reveal their cellular structure free from distortion and contraction. This method appears to be a distinct improvement in the technique of embedding tissues in paraffin. Experiments with amyl alcohol showed that this can be used in place of butyl alcohol, but as amyl alcohol will not mix with 50 per cent. ethyl alcohol in water, it is necessary to pass the tissue first through absolute ethyl alcohol, and for this reason it is not so good as butyl alcohol, and its use is not recommended.

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THE DEMONSTRATION OF A HITHERTO UNDESCRIBED TYPE OF CELL IN THE GLANDS OF THE STOMACH.

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Received for publication November 13th, 1924.

I HAVE recently published a preliminary note or letter (Twort, 1924[1]) on the demonstration of what I believe to be a hitherto undifferentiated type of cell in the glands of the stomach, and sections showing the cell were demonstrated at a recent meeting of the Physiological Society (1924[2]), when details of the staining methods employed were also given. In the course of the present paper it is proposed to give a fuller description of the cell, its distribution, and the methods by which it can be demonstrated.

The cell was first observed during some experiments which were being carried out on ultra-microscopic viruses on behalf of the Medical Research Council. The virus being investigated at the time was that which causes distemper of dogs. As is well known, in the early stages of distemper it is common to obtain an acute inflammatory condition of the mucous membrane of the stomach and of parts of the intestine, and it was these acute hæmorrhagic areas in the stomach of such cases that were being investigated. All attempts to cultivate the virus having failed, a detailed examination of sections was made, the sections being stained with the various modified stains of neutral red and light green (1924[3]), or with other special double dyes prepared for the purpose. It will not be necessary here to describe these experiments in detail; it will be sufficient to note that with none of the double dyes used and by none of the methods employed was it found possible to demonstrate anything definite which one could claim as being the virus of this disease. No parasite was demonstrated except the normal spirochæte and its round forms, which stain very densely with many of the neutral red-light green staining solutions.

In sections treated with the neutral red-light green stain the cells of the gastric glands stain well. The granules of the chief or peptic cells are coloured densely with the basic neutral red, and these for the most part completely hide the cytoplasm of the cell, which is tinged bluish-green. The chromatin of the nucleus also stains with the basic neutral red. In the case of the oxyntic or large parietal cells, these, except for the chromatin of the nucleus, which stains red, are coloured throughout a bluish green. The granules in their substance take on the same tint, although it is interesting to note that in delicately stained sections it is not uncommon to observe in addition a certain number of scattered minute granules which are coloured red. The number of granules and the intensity of their staining with this dye in both types of cell vary according to their situation and to the activity of the glands. In addition to these two types of cell will be seen some rather small cells with a large round or slightly oval nucleus which usually stains less densely than the nucleus of the peptic or oxyntic cell (see plate). The protoplasm of these cells stains a characteristic grass-green colour; moreover, the cells are crowded with similar staining granules, which may be tipped with a fine point of red coloration. The cells in shape and size most closely resemble

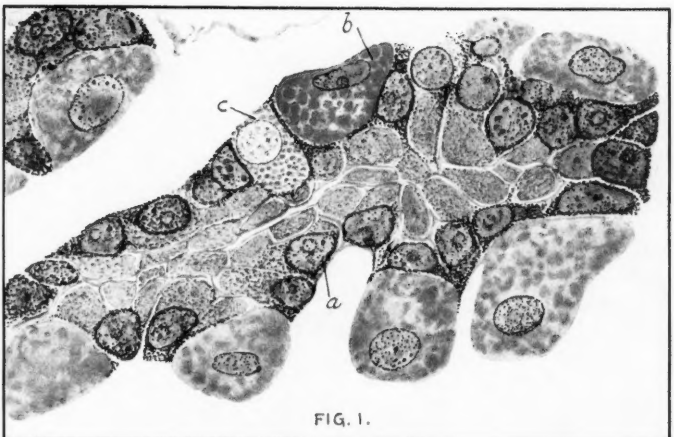

FIG. 1.

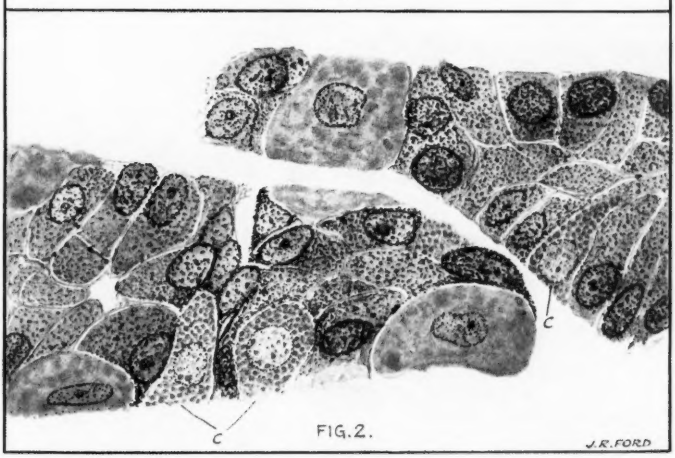

FIG. 2.

Two sections of mucosa from the greater curvature of dog's stomach, showing (*a*) peptic cell, (*b*) parietal cell, (*c*) the new cell described in this paper.

The tissue was fixed in 10% formalin-Müller, washed, treated with 60% alcohol, passed through several changes of normal butyl alcohol during 3 hours, and then through several changes of paraffin.

The sections were stained with neutral red - light green double stain prepared with 60% normal propyl alcohol.

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the peptic cells. They usually occur singly and are wedged in between the peptic cells, or more rarely are situated between the peptic and the oxyntic cells. They are most numerous in the deeper parts of the glands. The grass-green coloration taken on by these cells is quite distinct from the bluish-green of the oxyntic cells, while the presence of an abundance of grass-green granules and the delicately staining large round nucleus give them a characteristic appearance which is distinct from all other cells present.

It has been suggested that the granules of these cells might represent an intracellular stage in the life-history of some parasite, but their appearance lends no support to such a hypothesis. Moreover, experiments have shown that the cell may be found in such widely distinct species of animals as dogs, rabbits and sheep, and in animals of all ages and in those that are apparently free from disease, so that they can have no connection either with the virus of distemper or with the spirochæte to be found in the normal dog's stomach. In the case of dogs and rabbits, which have been the chief animals examined, the cells can be found from the cardiac to the pyloric end of the stomach, although it is possible that they may be more numerous in certain areas than in others, being in fact patchy in their distribution. In man only a few poor examples have been found, probably due to the fact that the only specimens that were available for examination were those obtained from the post-mortem room many hours after death, when the glands were becoming more or less disintegrated. The possibility that the cell might represent an oxyntic or a peptic cell in some stage of its development or activity was also considered, but in view of their well-defined characters, and the absence of any cell showing intermediate characters, it seems certain that this suggestion can be excluded. Moreover the cells are no less well defined in starving animals than in those in which digestion in some stage is taking place.

When using the neutral red-light green double stain for demonstrating these cells, it is advisable to arrange the staining so that the basic part of the dye does not stain too densely, or the cells may be largely masked by the surrounding peptic cells and their densely staining granules. For full details of the use of this stain the reader is referred to my papers dealing with this subject. The allyl alcohol modification of the stain, followed by differentiation with 60 per cent. ethyl alcohol, probably gives the best results.

It is probable that these cells have some special function to perform in connection with digestion. It may be that their secretions play a part in activating the pepsinogen, or in the production of the hydrochloric acid, or, on the other hand, they may secrete rennin or some other enzyme. No experiments have been carried out to test these points, although it may be of some interest to note that the cells appear to be more numerous in young animals than in old ones. The cell has not been found in Brunner's glands or in any other part of the body so far examined.

From what has been said it will be clear that these cells differ widely from the mucoid cells of Lim, and from the dark hæmatoxylin staining cells described by Stöhr as occuring in the glands of the pyloric end of the stomach.

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AN AUTOMATIC FILLER.

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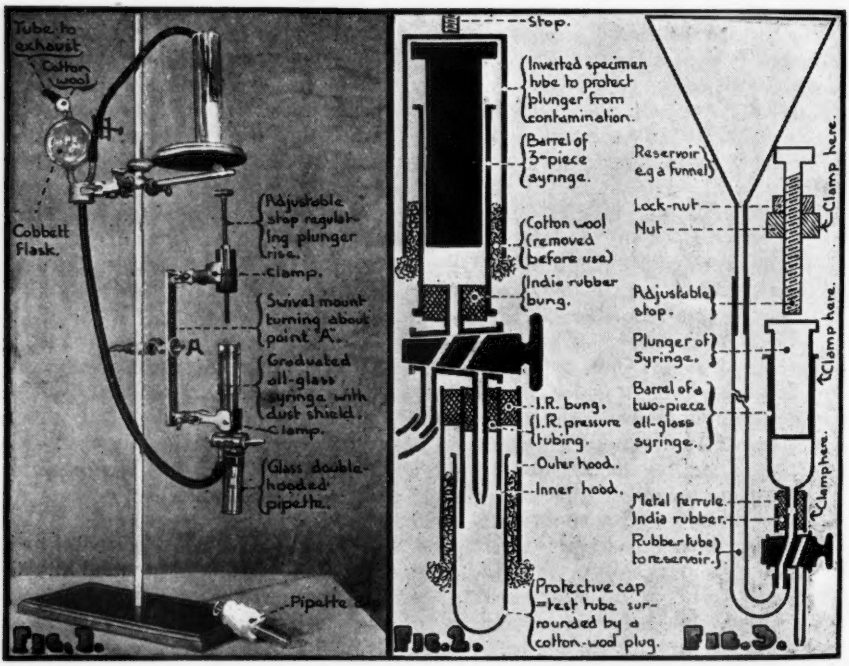
THE need for a ready means of measuring and delivering standard quantities of liquids becomes ever more pressing in laboratory work. A simple apparatus devised by the author some time ago has already been adopted by several institutions, and it is thought that it may prove of general use.



Briefly stated, the apparatus consists of a syringe into which fluid flows from an elevated reservoir, and which empties itself under the weight of the falling plunger.

The illustrations call for but little comment. Fig. 3 shows the simplest form—and the one which is most generally useful. Fig. 1 shows a form adapted for sterile work, as, *e.g.*, where a fluid needs to be filtered. Fig. 2 shows details of this filler, which is here represented ready for the autoclave.

Filter, Cobbett flask and filler, all having been connected up and then autoclaved together, are arranged as in Fig. 1. When the filtered fluid has passed into the Cobbett flask, the filler is clamped off and then the suction tube is disconnected. The filler now comes into use. If air-bubbles appear in the syringe they are readily got rid of by first filling the filler, and then turning it and its mount about the swivel. This brings the nozzle of the syringe uppermost, and when the plunger is pressed home the air passes into the reservoir tube and away up through the filtrate.

The double-hooded pipette is an advantage because the inner hood protects the delivery nozzle from all contact with test-tubes, and it ensures the fluid's being cleanly delivered, thereby eliminating sticking plugs.

With care the filler can be worked without the dust-shield with but little risk of contamination. When the fluid in the reservoir becomes insufficient to force up the plunger, the tubing can be finished by lifting the plunger by hand.

A good all-glass syringe is best. Metal plungers seldom work well. The 5 c.c. and 10 c.c. sizes are satisfactory. A very thin film of liquid paraffin well rubbed into the surface of the plunger and barrel will make for smoother working.

Once set, the accuracy of the filler is greater than that of an ordinary hand pipette skilfully used. If the syringe gradations are not sufficiently reliable the filler may be easily calibrated. A head of pressure greater than 18 inches is usually of no advantage.

The filler was originally designed for tubing culture media, and has been found to economize both material and time. Melted agar and gelatine are readily tubed by its means, and provided that fresh hot supplies are available, it will continue working indefinitely without clogging.

Acknowledgment is due to Dr. Purdy and to Mr. Barnard's department for the illustrations.

A NOTE ON HÆMOLYSIN AND HÆMAGGLUTININ WITH REFERENCE TO THE WASSERMANN REACTION.

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From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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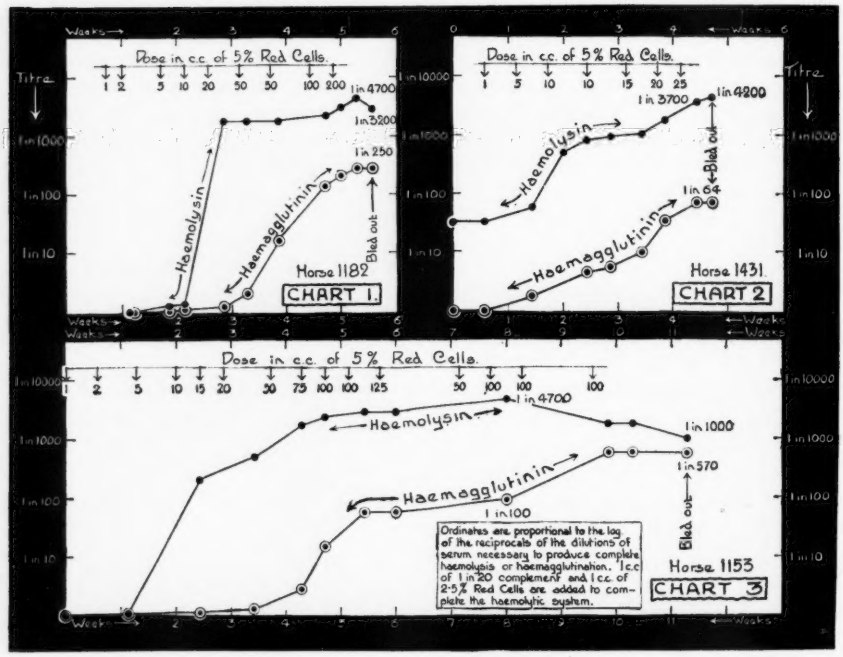
THE sequence of events observed in a horse or other animal immunized with sheep's red cells for the production of hæmolysin (amboceptor) has a practical bearing on the technique of the Wassermann and other complement-fixation tests.

Chart 1 illustrates the typical response of a horse injected with sheep's cells. Three separate phenomena connected with immunization are recorded on the chart, viz.—

(1) the production of hæmolysin (Bordet, 1898; Ehrlich and Morgenroth, 1899),
(2) the production of hæmagglutinin, and
(3) the gradually increasing tolerance of the animal to the injections, *i.e.* the development of immunity in the true sense. This is indicated, though only roughly, by the dosage of red cells recorded on the chart, since the reaction produced by each dose suggested that it was practically the maximum which could be tolerated by the animal at the time of injection without serious symptoms.


Dose in c.c. of 5% Red Cells.
Titre
Haemolysin
Haemagglutinin
Bled out
Horse 1182
CHART 1.
Horse 1431
CHART 2
Horse 1153
CHART 3
Ordinates are proportional to the log of the reciprocals of the dilutions of serum necessary to produce complete haemolysis or haemagglutination. 1 c.c of 1 in 20 complement and 1 c.c. of 2·5% Red Cells are added to complete the haemolytic system.

In our experience each progressive course of injections of red cells is followed by all three of these phenomena. It will be seen from the chart that, following the first small injection, there is a latent period of four to eight days, then a rapid rise of hæmolysin which may reach a titre of 1 in 1000 or more with hardly detectable agglutinin formation. A little later the agglutinins also begin to rise in titre, at first slowly, then much more rapidly.

Once the agglutinins begin to show rapid development the rate of increase of hæmolysin production tends to become relatively slow. Soon the hæmolytic titre shows no further rise in response to injections, and indeed it may begin to fall.

Chart 3 illustrates practically the same phenomena, but as a result of delay in making a full bleeding of the animal, the falling curve of the hæmolytic

titre and the rising curve of the hæmagglutinating titre tend to meet. As a consequence the serum from the final bleeding in this case proved unsuitable for hæmolytic tests, since hæmagglutination of the cells interfered with and masked hæmolysis.

As is well known, both horses and other animals occasionally contain naturally occurring anti-sheep hæmolysin in their serum. For example, Boerner (1915) reported the presence of natural anti-sheep hæmolysin in the serum of four of a series of two hundred horses which he examined; we have found it in two of a series of one hundred. Horses may also contain diphtheria antitoxin in their serum prior to immunization. Glenny and Sudmersen (1921) have shown that this normal antitoxin indicates the presence of active immunity, and that the animals react with the "secondary stimulus" response. In their experience also these horses are the most suitable for immunization. On analogy one would expect a more ready response to injections with sheep's red cells when natural anti-sheep hæmolysin is present.

Ruth Gilbert (1922) obtained large quantities of high-titre serum from a mule which before immunization contained in its blood natural hæmolysin for sheep's cells. We also have recently immunized a horse whose serum was naturally hæmolytic for sheep's cells in a titre of 1 in 32, and found that this animal responded exceptionally well to its immunization (Chart 2). No naturally occurring agglutinin for sheep's cells was detectable in this horse.

The test hæmolytic system consisted of 1 c.c. of serum dilution (in this case 1 in 32), 1 c.c. of 5·0 per cent. washed and re-suspended sheep's red cells, and 1 c.c. of 5 per cent. guinea-pig serum (complement) of normal activity. Readings were made after one hour in the water-bath at 37°C.

Cells washed with Ringer's fluid were used throughout for injection, as these appeared to cause less reaction than cells washed with normal saline.

The following series of red-cell doses was given: 1, 5, 10, 10, 15, 20 and 25 c.c., the interval between each dose being from three to five days. The horse showed remarkably little reaction to the gradually increasing doses of the injections, and only once had the same dose to be repeated owing to a rise in temperature. Within five weeks, after 86 c.c. in all of red cells had been injected, the hæmolytic titre had reached 1 in 4200 and the hæmagglutinative 1 in 64.

The details of the immunization of this horse are shown in Chart 2.

In connection with the occurrence of natural hæmolysin in animals, we thought it of interest to determine whether the natural hæmolysin present in this horse was of an isophile or heterophile type (Forssman, 1911; Friedemann, 1917). Following Taniguchi's technique (1921), we were unable to show any absorption of the hæmolysin by alcoholic and watery extracts of guinea-pig kidney and horse heart (heterophile antigens). Moreover, the hæmolysin present before immunization showed similar sensitiveness to heat as a hæmolytic horse-serum of the same titre of known isophile origin. This indicates that the natural hæmolysin present in this case was isophile in type. We are thus faced with a similar problem as in the case of the naturally occurring diphtheria antitoxin of the horse, where up to now it has been impossible to demonstrate the presence of any specific stimulus for its production.

We would like to emphasize the utility of a careful control of the injections by frequent experimental bleedings and the charting of the results. Inspection of the charts shows at once the all-important relation between the hæmolytic and the hæmagglutinating titres. The value of a hæmolytic serum obviously varies not only directly as its hæmolytic titre, but also inversely as its hæmagglutinating titre. When the agglutinating activity is small, it is easy to prepare "sensitized cells" containing a large number of doses of the hæmolysin, and although a system containing, say, two and a half M.H.D. of hæmolysin is probably quite as serviceable in the Wassermann reaction as one containing five or ten, many workers prefer a system with a large excess of hæmolysin.

The aim in immunization with red cells is therefore to produce hæmolysin with as little agglutinin as possible, and for this the following three methods suggest themselves:

1. To separate the hæmolysin from the hæmagglutinin in the serum.
2. To separate the hæmolytic antigen from the hæmagglutinative antigen.
3. So to arrange the course of immunization of the animal and the time of bleeding that the serum is strong in hæmolytic and weak in agglutinative properties.

With regard to the first method—separation of the serum—we have found, in agreement with most other observers, that lysin and agglutinin cannot be separated by fractionation of the serum proteins by the usual methods. Both lysin and agglutinin reside in the same fractions, viz. the globulins.

Attempts at partial absorption by red cells and filtration through various grades of filters have never in our hands been successful in separating the two elements.

With regard to 2, there seems to be no means of producing with certainty hæmolysin without hæmagglutinin by any antigenic modification so far employed (*cf.* Stewart, 1904).

The work of Coulter (1921) has shown that the same pH, viz. 5·3, corresponds with—

1. the iso-electric point of the serum globulin;
2. the reaction at which maximum agglutination of sensitized red cells takes place; and
3. the reaction at which the largest amount of hæmolysin is absorbed by cells.

This suggests a fundamental colloidal similarity between the lysin and the agglutinin, and it does not encourage us to hope for any chemical separation of the two elements.

We are left, then, with the third method, that of careful interpretation of the course of immunization and bleeding at the critical time, and it is this method that the charts illustrate. It seems probable that agglutinins correspond with the end immunity of the animal to the poison of the heterologous cells, and once they are available no further effort is made to produce hæmolysin. The classical methods of antitoxic immunization, which aim at the production of maximum tolerance to a foreign poison, do not therefore necessarily hold with regard to hæmolysin.

It may, then, be concluded that every high-grade hæmolytic serum is also to some extent hæmagglutinative. This, of course, is a matter of every-day importance in the Wassermann and other hæmolytic tests.

If a large excess of hæmolysin is used to sensitize cells, it may well happen that agglutination, more or less marked, may take place in the system of sensitized cells. If these cells are made up and used immediately, the agglutination may not be noticed, but even then it may upset the subsequent tests, causing blurred readings. The cells may, of course, appear agglutinated to the naked eye, and this is particularly likely to happen if the sensitized cells are kept overnight or for some time in a warm place. If they are shaken up they may appear to the naked eye to be completely re-suspended. The following experiment illustrates the danger of using cells shaken up after agglutination:

1 c.c. of the following dilutions of a hæmolysin (hæmolytic titre, 1 in 2000) and 1 c.c. of 5 per cent. red cells were put together. The usual method of 20 per cent. dilutions of hæmolysin was used, the hæmolysin in each tube being 20 per cent. more dilute than in the preceding tube. The agglutination titre is recorded below:

(Sensitising)—Agglutination Systems.

	$\frac{1}{50}$	$\frac{1}{62}$	$\frac{1}{78}$	$\frac{1}{97}$	$\frac{1}{120}$	$\frac{1}{150}$	$\frac{1}{187}$	$\frac{1}{234}$	$\frac{1}{292}$	$\frac{1}{365}$	$\frac{1}{456}$
I. 3 hours at 4° C.	(A)	a	a	a	o	o	o	o	o	o	o
II. 3 hours at 37° C.	A	A	A	(A)	(A)	(A)	a	a	a	o	o
III. 24 hours at 4° C.	A	A	A	A	(A)	a	a	a	o	o	o
IV. 24 hours at 15° C.	A	A	A	A	A	A	(A)	(A)	a	o	o

A = appearance of complete agglutination.
(A) = appearance of partial agglutination.
a = appearance of traces of agglutination.
o = no agglutination.

Each tube was shaken up until the cells appeared entirely re-suspended to the naked eye. 1 c.c. quantities of the mixtures were withdrawn from each tube, and to these 1 c.c. of 1/20 complement and 1 c.c. of saline were added. The following are the readings of the hæmolysis at the end of one hour in the water-bath at 37° C.:

Hæmolytic Systems.

	$\frac{1}{50}$	$\frac{1}{62}$	$\frac{1}{78}$	$\frac{1}{97}$	$\frac{1}{120}$	$\frac{1}{150}$	$\frac{1}{187}$	$\frac{1}{234}$	$\frac{1}{292}$	$\frac{1}{365}$	$\frac{1}{456}$
I	3 A	3 (A)	3 (A)	3 (A)	3 (A)	3	3	3	3	3	3
II	3 A	3 A	3 A	3 A	3 A	3 A	3 A	3 A	*3·2*	3	3
III	3 A	3 A	3 A	3 (A)	3 (A)	3 (A)	3 (A)	3 (A)	*3·2*	*3·2*	3
IV	2 A	2 A	2 A	2 A	2 A	2 A	2 A	2 A	2 A	2	2

3 = appearance of complete hæmolysis.
3·2 = appearance of almost complete hæmolysis.
2 = appearance of partial hæmolysis.
A = appearance of complete agglutination.
(A) = appearance of partial agglutination.

It will be seen that at dilutions 1/234 to 1/365 little or no agglutination is visible in the first part of the experiment. In the second part of the experiment after the addition of complement, though at these dilutions there is a large excess of hæmolysin present, for the titre was 1 in 2000, yet an appearance indistinguishable from incomplete hæmolysis was present in certain tubes

(shown in lines *II*, *III* and *IV* of the tables) between the stage of visible agglutination and complete hæmolysis. This is no doubt the explanation of the blurred readings which sometimes occur in Wassermann work when too large a dose of hæmolysin is used without regard to its agglutination titre. Similar results were obtained with rabbit hæmolysin.

With the ice-box method of fixation this point becomes of special importance, as the sensitized cells may have to be kept for twenty-four hours or so. As one can see by the tables, both the time and temperature factors are important.

In the initial testing of a hæmolysin it is always well to estimate its agglutinative as well as its hæmolytic titre under the conditions of concentration, time and temperature that will be required for the particular Wassermann method used. One such estimation only need be made on each batch of serum, as the hæmagglutinin is much more stable than the hæmolysin.

Too great an excess of hæmolysin should not, of course, be used in the Wassermann test, for the concentration of the hæmolytic serum might then be such that its hæmagglutinating properties are not inhibited. No hæmolytic serum of high titre in our experience is ever completely free from hæmagglutinins.

SUMMARY.

1. In order to obtain economically a large yield of useful hæmolysin in horses, it is important to control the immunization by frequent experimental bleedings, the results of which are charted in such a way as to show the relative production of hæmolysin and hæmagglutinin.

2. When the hæmagglutinin begins to show a marked rise relative to the hæmolysin it is advisable to bleed the horse for a maximum yield, as by delay the hæmolytic and hæmagglutinating titres may so approximate as to render the hæmolysin practically useless for hæmolytic work.

3. A horse showing natural hæmolysin for sheep's cells before immunization ran a particularly favourable course of immunization. The natural hæmolysin in this horse had the properties of isophile antibody (Forssman, Friedemann and Taniguchi).

4. Blurred hæmolysis where complete hæmolysis should be present in the titrations of the Wassermann reaction may be due to undue hæmagglutinating activity of the hæmolytic serum, *e. g.* by using too great a concentration of a high titre hæmolytic serum in the system of sensitized cells.

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INDEX TO VOL. V.

ALLAN, F. N., BOWIE, D. J., MACLEOD, J. J. R., and ROBINSON, W. L., behaviour of depancreatized dogs kept alive with insulin, 75
ALLISON, V. D., the effect of the administration of vaccines on the lysozyme content of tissues and secretions, 165
ANDREWES, C. H., the nature of the difference between the bilirubins of obstructive and hæmolytic jaundice, 213
Antibodies, reputed chemical stimulation of, 18
Anti-dysentery serum, standardization of, 100
ARKWRIGHT, J. A., the source and characteristics of certain cultures sensitive to bacteriophage, 23
—— and GOYLE, A. N., the relation of the "smooth" and "rough" forms of intestinal bacteria to the "O" and "H" forms of Weil and Felix, 104
AYLING, T. H., an automatic filler, 354

Bacilli, acid-fast, group specificity of antigens, 171
B. acidophilus odontolyticus, characters and distribution of, 175
B. coli, bearing of fixation areas on the pathogenicity of, 123
B. dysenteriæ, standardization of anti-serum, 100
B. influenzæ, growth requirements of, 69
B. tuberculosis, determination of infections by silica, 158
—— group specificity of antigens, 171
Bacteria, intestinal, "smooth" and "rough" forms of, 104
Bacterial antigens, properties of, prepared by various methods, 185, 205
—— invasion, effect of radium and X-rays, 220
Bactericidal power of blood, reduction of, by decalcifying agents, 47
—— —— effect of radiation on, 54
—— property of milk, 271
Bacteriophage, characteristics of cultures sensitive to, 23
—— reproduction in a synthetic medium, 40
BAKER, S. L., DICKENS, F., and DODDS, E. C., the distribution of insulin in human and other animal tissues, with a description of a micro-method for the estimation of insulin in tissues, 327
BANNERMAN, R. G., variations in the number of blood-plates associated with a common cold, 16
BENIANS, T. H. C., further experiments with fixation areas, bearing on the pathogenicity of *Bacillus coli* in peritoneal infections, 123
Bilirubin in obstructive and hæmolytic jaundice, 213
BOWIE, D. J., see *Allan, F. N.*
BURNET, F. M., observations on the agglutinins in typhoid fever, 251

CAMPBELL, A., and HILL, L., the effects of light upon leucocytes and blood-vessels in the mesentery of the living animal, 317
CHAMBERS, HELEN, and SCOTT, GLADWYS M., experiments on immunity to tumour growth, 1
Cholesterol of blood in malignant disease, 293
Cirrhosis, production of, by manganese, 92
CLARKE, J. K., on the bacterial factor in the ætiology of dental caries, 141
COLEBROOK, L., EIDINOW, A., and HILL, L., the effect of radiation on the bactericidal power of the blood, 54
—— and STORER, E. J., on the reduction of the bactericidal power of the blood which is effected by adding to it citrate of soda and other decalcifying agents; and on the question whether blood so treated should be employed for immuno-transfusion, 47
COWAN, MARY L., variation phenomena in streptococci; further studies in virulence and immunity in mice and rabbits, 226
CRAMER, W., on the glycogenic function of the liver and its endocrine control, 128
—— and KINGSBURY, A. N., local and general defences against infections, and the effect on them of vitamin-deficiency, 300
CRICHTON, J. A., see *Telfer, S. V.*

CURRIE, A. N., the cholesterol of blood in malignant disease, 293

Dental caries, ætiology of, 141, 175
DICKENS, F., see *Baker, S. L.*, and *Dodds, E. C.*
DOBSON, J. F., see *Stewart, M. J.*
DODDS, E. C., see *Baker, S. L.*
——— and DICKENS, F., some observations on the properties and preparation of insulin, with special reference to the picrate-acetone method of preparation, 115

EAGLES, G. H., the significance of serological grouping of hæmolytic streptococci, with special reference to *Streptococcus scarlatinæ*, 199
EIDINOW, A., see *Colebrook, L.*
Endocrine control of the glycogenic function of the liver, 128

FERRY, N. S., and FISHER, L. W., studies on the immunizing properties of bacterial antigens prepared after various methods, 185, 205
FILDES, P., the growth requirements of hæmolytic influenza bacilli, and the bearing of these upon the classification of related organisms, 69
FINDLAY, G. M., the experimental production of biliary cirrhosis by salts of manganese, 92
FISHER, L. W., see *Ferry, N. S.*
FLEMING, A., on the accuracy of measurement of small volumes of fluid with a capillary pipette, 148

Glycogenic function of the liver, 128
GOYLE, A. N., see *Arkwright, J. A.*
GYE, W. E., and PURDY, W. J., the poisonous properties of colloidal silica, 238

Hæmagglutinin, relation to the Wassermann reaction, 355
Hæmolytic influenza bacilli, growth requirements of, 69
HANSSEN, F. S., the bactericidal property of milk, 271
HARTLEY, P., the effect of radiation on the production of specific anti-bodies, 306
HILL, L., see *Campbell, A.*, and *Colebrook, L.*
Hodgkin's disease, inoculation experiments in monkeys, 65
H-ion concentration, determination of, in pathological plasma, 13

Immuno-transfusion, use of decalcified blood in, 47
Insulin, behaviour of depancreatized dogs kept alive with, 75
——— distribution of, in tissues, 327
——— estimation of, 327
——— preparation of, 115
Intracranial pressure, effect of increased, on medullary centres, 281

JAMES, W. W., see *McIntosh, J.*
Jaundice, bilirubins of, 213
JOHNSTON, MARY E., the mechanism of the immunity to anti-platelet serum, 261

KETTLE, E. H., the demonstration by the fixation abscess of the influence of silicæ in determining *B. tuberculosis* infections, 158
KINGSBURY, A. N., see *Cramer, W.*, *McIntosh, J.*, and *Mottram, J. C.*

LAZARUS-BARLOW, P., see *McIntosh, J.*
LAZARUS-BARLOW, W. S., and PARRY, R. H., the liver and certain other tissues in relation to acquired immunity towards Jensen's rat sarcoma, 34
LEDINGHAM, J. C. G., the reaction of the skin to vaccinia virus, 332
LEPPER, ELIZARETH H., the reproduction of bacteriophage when the sensitive organism is grown in a synthetic medium, 40
Lysozyme content, effect of the administration of vaccines on, 165

MCINTOSH, J., JAMES, W. W., and LAZARUS-BARLOW, P., an investigation into the ætiology of dental caries: II. The biological characteristics and distribution of *B. acidophilus odontolyticus*: III. Further experiments on the production of artificial caries, 175
——— and KINGSBURY, A. N., on the reputed chemical stimulation of anti-body production, 18
MACLEOD, J. J. R., see *Allan, F. N.*
Manganese, production of biliary cirrhosis by, 92
MARRACK, J., and SMITH, F. C., colorimetric determination of pH in pathological plasma by Cullen's method, 13
Measurement of small volumes of fluid, 148
Milk, bactericidal property of, 271
——— influence of mineral deficiency in, on skeletal growth, 84
MOTTRAM, J. C., and KINGSBURY, A. N., some researches into the action of radium and X-rays correlating the production of intestinal changes, thrombopœnia and bacterial invasion, 220

O'BRIEN, R. A., see *Sudmersen, H. J.*
Œdema in cattle, 313
OKELL, C. C., and PARISH, H. J., a note on hæmolysin and hæmagglutinin with reference to the Wassermann reaction, 355

Paraffin, embedding tissues in, 350
PARISH, H. J., see *Okell, C. C.*
PARRY, R. H., see *Lazarus-Barlow, W. S.*

PERKINS, R. J., see *Twort, C. C.*
PURDY, W. J., see *Gye, W. E.*
Platelets, immunity, to anti-serum, 261
——— reduction of, effect of radium and X-rays, 220
——— variations of, in a common cold, 16
——— effect on the bactericidal power of the blood, 54

Radiation, effect on leucocytes and blood-vessels in frog's mesentery, 317
——— effect on the production of specific antibodies, 306
Radium, effect of, on thrombopœnia, 220
ROBINSON, W. L., see *Allan, F. N.*
RUNGE, B. F., see *Sudmersen, H. J.*

Scarlatina, serological grouping of streptococci, 199
SCOTT, GLADWYS M., see *Chambers, Helen.*
Silica, influence of, in determining *B. tuberculosis* infections, 158
——— poisonous properties of colloidal, 238
SMITH, F. C., see *Marrack, J.*
Stain, modifications in the preparation of neutral red–light green, 350
STAMMERS, A. D., œdema in South African cattle, 313
STEWART, M. J., and DOBSON, J. F., inoculation and implantation experiments in monkeys with glands from cases of Hodgkin's disease, 65
Stomach, new type of cell in glands of, 352
STORER, E. J., see *Colebrook, L.*
Streptococci, variation in, 226
Streptococcus scarlatinæ, serological grouping of, 199
SUDMERSEN, H. J., RUNGE, B. F., and O'BRIEN, R. A., standardization of anti-dysentery (Shiga) serum, 100

TELFER, S. V., and CRICHTON, J. A., the influence of mineral deficiency in goats' milk on the skeletal growth of kids, 84
TODD, E. W., see *Twort, C. C.*
TSUBURA, S., the effects of increased intracranial pressures on the medullary centres, 281
Tumours, immunity to growth of, 1
——— malignant, cholesterol of blood in, 293
——— relation of the liver to Jensen's rat sarcoma 34
TWORT, C. C., TODD, E. W., and PERKINS, R. J., studies on the group specificity of some antigens derived from acid-fast bacilli, 171
TWORT, F. W., further modifications in the preparation of neutral red–light green double stain, and an improved method of embedding tissues in paraffin, 350
——— the demonstration of a hitherto undescribed type of cell in the glands of the stomach, 352
Typhoid fever, agglutinins in, 251

Vaccinia virus, reaction of the skin to, 332
Vitamin-deficiency, effect of, on defence against infection, 300

Wassermann reaction, relation to hæm-agglutinin, 355

X-rays, effect of, on thrombopœnia, 220